# INVESTIGATION OF ANTIOXIDANT PROPERTIES AND ANTICARCINOGENIC EFFECTS OF ETHANOLIC EXTRACT FROM BARK OF SALIX AEGYPTIACA AND ITS FRACTIONS

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Submitted by **MÜŞERREF ŞEYMA CEYHAN** in partial fulfillment of the requirements for the degree of **Master of Science in Biology Department**, **Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Gülay Özcengiz _____
Head of the Department, **Biology**

Assoc. Prof. Dr. Sreeparna Banerjee _____
Supervisor, **Biology Dept., METU**

**Examining Committee Members:**

Assoc. Prof. Dr. A. Elif Erson-Bensan _____
Biology Dept., METU

Assoc. Prof. Dr. Sreeparna Banerjee _____
Biology Dept., METU

Prof. Dr. Arif Ahmet Başaran _____
Pharmacognosy Dept, Hacettepe University

Assoc. Prof. Dr. Mesut Muyan _____
Biology Dept., METU

Assist. Prof. Dr. Erhan Astarcı _____
Crop and Animal Production Dept., AIBU

**Date :** 06.02.2014

**I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.**

Name, Last Name: Müşerref Şeyma Ceyhan

Signature :

## ABSTRACT

INVESTIGATION OF ANTIOXIDANT PROPERTIES AND ANTICARCINOGENIC EFFECTS OF ETHANOLIC EXTRACT FROM BARK OF SALIX AEGYPTIACA AND ITS FRACTIONS

Ceyhan, Müşerref Şeyma

MSc., Department of Biology

Supervisor: Assoc. Prof. Dr. Sreeparna Banerjee

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Cancer is a multi-factorial disease that may arise as a consequence of different factors such as age, diet, and environmental factors as well as genetic makeup. Moreover, cancerous cells can develop certain strategies to evade immune system and develop resistance against chemotherapy drugs. Therefore, multi-targeted therapeutics have gained importance for both prevention of cancer and better prognosis.

Extracts from *Salix aegyptiaca* includes several active phytochemicals; phenolic compounds and flavonoids and therefore have the potential for use as herbal remedies. The aim of this study was to investigate antioxidant properties and anticarcinogenic effects of ethanolic extract from bark (EEB) of *Salix aegyptiaca* and its fractions in colon cancer. EEB was fractionated into different fractions by solvent solvent partitioning. The antioxidant property of EEB and its fractions were investigated by 1, 1-diphenyl -2-picryl hydrazyl (DPPH) free radical scavenging activity, total phenolic and flavonoid content assays. Effect of EEB and its active

fractions on cancer cell proliferation were evaluated by MTT and BrdU incorporation assays.

The ethyl acetate fraction showed the highest free radical scavenging activity (11 ± 1 μg/ml) and total phenolic content (29.6 ± 2 mg GAE/100 g dried weight). EEB had the highest total flavonoid content (47.5 ± 2 mg of EGCG equivalent/100 g dried weight) compared to its fractions. EEB, ethyl acetate and aqueous fractions decreased proliferation of cancer cells. Catechin, catechol and salicin were detected in high quantity in the ethyl acetate and aqueous fractions by tandem mass spectrometry (MS). Treatment of cancer cells with a combination of catechin and catechol had higher efficacy in reduction of proliferation than the individual pure compounds. EEB led to significant decrease in phosphorylation of ERK1/2, JNK1/2 and p38, but its active fractions were only functional in reducing the phosphorylation of ERK1/2.

To conclude, our data suggest that EEB and its active fractions could be thought of as a potent antioxidant and anticarcinogenic agent that may be used in complementary and alternative medicine.

Key words: *Salix aegyptiaca*, Colon cancer, Antioxidant, MAPK pathways

# ÖZ

SALIX AEGYPTIACA KABUĞUNDAN ELDE EDİLEN ETANOLİK ÖZÜTÜN VE ONUN FRAKSİYONLARININ ANTİOKSİDAN ÖZELLİĞİNİN VE ANTİKARSİNOJENİK ETKİLERİNİN İNCELENMESİ

Ceyhan, Müşerref Şeyma
Yüksek Lisans, Biyoloji Bölümü
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Kanser genetik yapının yanısıra yaş, diyet, ve çevresel etmenler gibi birçok farklı faktörün sonucunda ortaya çıkan multi-faktöriyel bir hastalıktır. Ayrıca, kanserli hücreler bağışıklık sisteminden kaçmak ve kemoterapi ilaçlarına karşı direnç göstermek için belirli stratejiler geliştirmişlerdir. Böylece çok hedefli terapötikler hem kanserden korunma hem de daha iyi prognoz için önem kazanmıştır.

*Salix aegyptiaca* özütü fenolik bileşikler ve flavonoidler gibi birçok aktif fitokimyasalları içerir ve bu yüzden bitkisel ilaç olarak kullanılma potansiyeli vardır. Bu çalışmanın amacı *Salix aegyptiaca* kabuğundan elde edilen etanolik özütün (EEB) ve onun fraksiyonlarının antioksidan özelliğinin ve MAPK yolağıyla antikarsinojenik etkilerinin kolon kanserinde incelenmesidir. EEB çekme yöntemi ile farklı fraksiyonlara ayrılmıştır. EEB ve fraksiyonlarının antioksidan özelliği DPPH serbest radikalleri uzaklaştırma aktivite, total flovanoid ve total fenolik içerik deneyleri ile araştırılmıştır. EEB ve onun aktif fraksiyonlarının kanserli hücre

çoğalmasının üzerindeki etkisi MTT ve BrdU katılım deneyleri ile değerlendirilmiştir.

En yüksek serbest radikalleri uzaklaştırma aktivitesi (11 ± 1 μg/ml) ve total fenolik içeriği (29.6 ± 2 mg gallik asit eşleniği / 100 gram kurutulmuş ekstrakt) etil asetat fraksiyonu göstermiştir. Fraksiyonlarına kıyasla en yüksek total flavonoid içeriğe (47.5 ± 2 mg EGCG eşleniği / 100 gram kurutulmuş ekstrakt) EEB sahiptir. EEB, etil asetat ve su fazı fraksiyonları kanser hücrelerinin çoğalmasını azaltmıştır. Ardışık kütle spektrometre yöntemi ile etil asetat ve sulu faz fraksiyonlarında yüksek miktarda katekin, katekol ve salisin belirlenmiştir. Katekin ve katekol kombinasyonunun kanserli hücrelere uygulanması saf bileşiklere kıyasla hücre çoğalımının azalmasında daha yüksek bir etkiye sahiptir. ERK1/2, JNK1/2 ve p38 fosforilasyonunda önemli azalma EEB ile sağlanmıştır, fakat aktif fraksiyonları sadece ERK1/2 fosforilasyonunda azalmaya neden olmuştur. Sonuç olarak, verilerimiz gösteriyor ki EEB ve onun fraksiyonları tamamlayıcı ve alternatif tıpta güçlü bir antioksidan ve antikarsinojenik ajan olarak kullanılabilir.

Anahtar kelimeler: *Salix aegyptiaca*, Antioksidan, Kolon kanseri, MAPK yolakları

***To My Family***

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# CHAPTER 1

# INTRODUCTION

## 1.1 Antioxidants

An antioxidant is simply defined as any substance that has the ability to delay, prevent or remove oxidation reactions of a molecule (Halliwell, 2007). Antioxidants are used commonly in daily life in order to improve health and extension of life expectancy. Moreover, some antioxidants are utilized in the food and cosmetics industry as preservatives (Gamboni et al., 2013).

Antioxidants have different ways to prevent oxidative damage such as inhibition of ROS production, scavenging of ROS and induction of antioxidant defenses (Khlebnikov et al., 2007). The antioxidant defense system is based on the donation of an electron to free radicals and elimination of ROS/RNS initiators (Choudhari et al., 2013). Therefore, antioxidants can prevent free radical formation by scavenging the initial radicals and chelating metals. In addition, antioxidants can convert more reactive species to less harmful, stable products such as the reduction of hydrogen peroxide to water.

Repair mechanisms are also involved in antioxidant defense system to eliminate oxidized proteins and repair damaged DNA by proteinases, proteases, peptidases and nucleases (Lobo et al., 2010).

### 1.1.1 Classification of Antioxidants

Antioxidants are divided into several groups according to their action mechanisms, molecular structures and sources (Carocho and Ferreira, 2013). The classification of antioxidants helps in understanding their importance and actions in antioxidant defense systems (Figure 1.1)

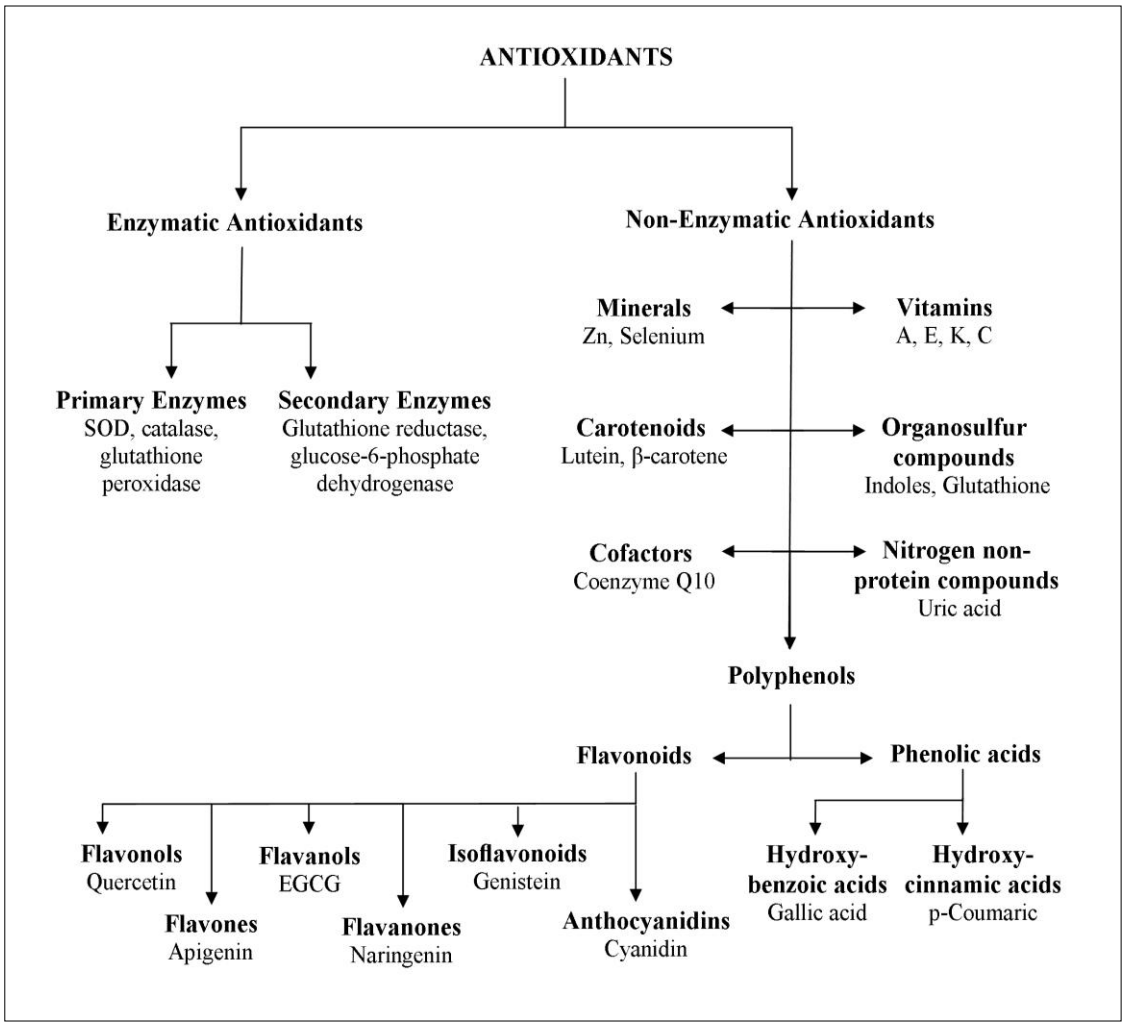


**Figure 1. 1** Classification of antioxidants. Retrieved from (Carocho and Ferreira, 2013).

#### 1.1.1.1 Natural Antioxidants

Antioxidants are classified into two groups: enzymatic antioxidants and non-enzymatic antioxidants. Primary and secondary enzymatic antioxidants are classified within the scope of enzymatic antioxidants. Glutathione peroxidase, catalase, and superoxide dismutase enzymes are the primary enzymes (Figure 1.2). Glutathione reductase and glucose-6-phosphate dehydrogenase are classified as secondary enzymes. Vitamins, carotenoids, minerals, organosulfur compounds and phytochemicals are placed in non-enzymatic antioxidants (Carocho and Ferreira, 2013).

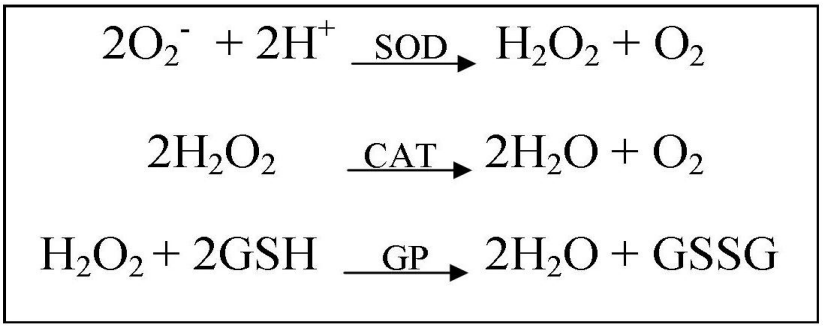


**Figure 1. 2** Reaction mechanisms that enzymatic antioxidants are involved in. Retrieved from (Kulbacka, 2012).

#### 1.1.1.1.1 Enzymatic Antioxidants

**Superoxide Dismutase**

SOD enzyme was isolated by Mann and Keilin in 1938, and its ability to enzymatically eliminate superoxides was discovered by McCord and Fridovich in 1968 (Halliwell and Gutteride, 1989). There are two different SOD enzymes based on their locations and the metal prosthetic groups. Copper-zinc-containing superoxide dismutase (CuZnSOD), which has two subunits containing one active site for each metal, is found in the mitochondria and cytoplasm. Manganese-containing superoxide dismutase (MnSOD) is a mitochondrial matrix enzyme. SOD enzymes convert two superoxide radicals to one hydrogen peroxide (Milbury and Richer, 2008).

**Catalase**

In 1901, Loew identified catalase enzyme (Nicholls, 2012), which is found in peroxisomes, as an important enzyme for the conversion of $H_2O_2$ to $H_2O$ and $O_2$ (Kulbacka, 2012).

**Glutathione Peroxidase**

Oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) is carried out by glutathione peroxidase (GPx) enzyme to remove hydrogen peroxide from cell. Selenium is employed in oxidation-reduction reactions by GPx enzyme as a cofactor. The GSH to GSSG ratio should be high in normal cells thus; glutathione is regulated

by a glutathione system that involves glutathione reductase, glutathione peroxidases, and glutathione S-transferases enzymes (Lobo et al., 2010; Marty et al., 2009).

**Glutathione Reductase and Glucose-6-Phosphate Dehydrogenase**

Under normal conditions, reduced glutathione (GSH) level is elevated relative to oxidized glutathione (GSSG) by glutathione reductase enzyme, which provides to recycle GSH in the cellular glutathione system (Figure 1.3) (Wu et al., 2004). Reduction of glutathione is a NADPH driven reaction provided by Glucose-6-Phosphate Dehydrogenase enzyme from oxidative pentose phosphate pathway (Halliwell and Gutteride, 1989; Dodson et al., 2013). These two enzymes play important roles in redox reactions of the glutathione system.

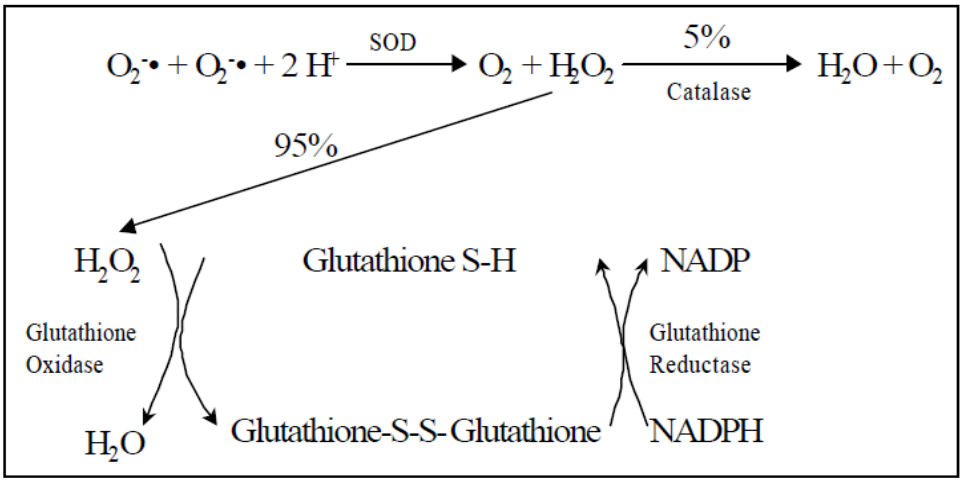


**Figure 1. 3** Summary of redox reactions in antioxidant defense system. Retrieved from (Wall, 2000).

#### 1.1.1.1.2 Non-Enzymatic Antioxidants

Examples of non-enzymatic antioxidants are endogenous compounds such as vitamin A, uric acid, coenzyme Q10 and glutathione. Generally, non-enzymatic antioxidants are taken from dietary sources to sustain antioxidant defense (Milbury and Richer, 2008; Ratnam et al., 2006). If the antioxidant system becomes weak due to deficiency of dietary antioxidants, it can lead to many diseases such as cardiovascular diseases (Madamanchi et al., 2005), neurodegenerative diseases (Darvesh et al., 2010) and cancer (Perse, 2013).

Vitamin C and E, selenium, β-carotene, lutein and polyphenols are examples of exogenous antioxidants. These phytochemicals are secondary metabolites of plants that evolved for defense against pathogens and for reproduction. Polyphenols constitute a high proportion of dietary antioxidants and are divided into subclasses such as phenolic acids, flavonoids, stibenes, coumarins and tannins (Liu, 2004). Hydroxybenzoic acids and hydroxycinnamic acids are classified as phenolic acids and vannilic, syringic, gallic acids are some examples of hydroxybenzoic acids. p-coumaric and ferulic acid are examples of hydroxycinnamic acids. These phenolic compounds have free radical scavenging and metal chelating ability, which therefore act as significant antioxidants against free radicals (Carocho and Ferreira, 2013; Krimmel B., 2010).

Flavonoids, which are also plant-derived antioxidants, are classified as flavonols, flavones, flavanols (catechins), flavonones, anthocyanidins and isoflavonoids. These compounds have a common structure: two aromatic rings and one oxygenated heterocyclic carbon ring (Figure 1.4) (Liu, 2004).

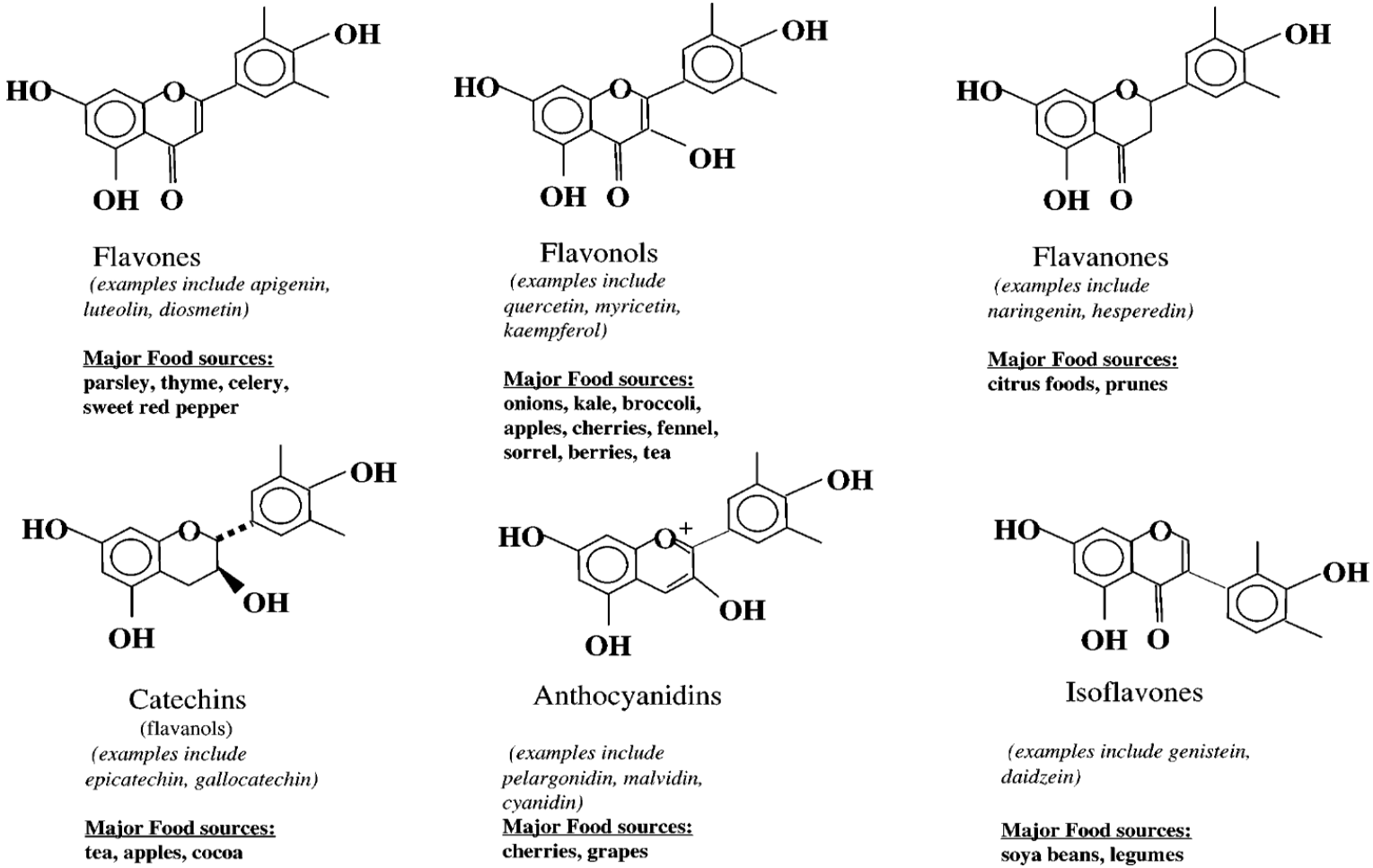


**Figure 1. 4** Major classes of flavonoids. Retrieved from (Ross and Kasum, 2002)

A high number of phenolic hydroxyl groups provide high antioxidant ability by using different defense mechanisms including activation of antioxidant enzymes, inhibition of oxidases, scavenging of ROS and metal chelation (Prochazkova et al., 2011). Flavonols (quercetin, kaempferol, and myricetin) are found in fruits and vegetables that have inhibitory effect on (CYP P450) drug metabolizing enzyme system. Flavones (luteolin and apigenin) can isolated from cereals and herbs that also inhibit CYP2C9 enzyme (Si et al., 2009). Flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate) are provided mainly from tea and fruits as dietary source and these compounds differ from other flavonoids in terms of lacking ketone group in their chemical structure (Hollman and Arts, 2000). Flavanones (naringenin, hesperidin) are generally found in citrus fruits (Erlund, 2004), anthocyanidins (cyanidin, pelargonidin) are mostly taken from berries and grapes, which provide UV protection and reduce photooxidative stress in plant cells (Gould, 2004), and isoflavonoids (genistein) are supplied from legumes (Cook and Samman, 1996).

The anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, antiviral, and anticarcinogenic activities of flavonoids have been shown in many studies (Middleton E.Jr., 2000). Flavonoids have many effects on different vital cell signaling pathways such as phosphoinositide 3-kinase (PI3-kinase), Akt/PKB, tyrosine kinases, protein kinase C (PKC), and Mitogen Activated Protein (MAP) kinases as well as antioxidant defense system (Chrubasik et al., 2004). For instance, Spencer et al. showed the reduction in phosphorylation levels of Akt/PKB and ERK proteins in the presence of quercetin (Williams et al., 2004; Spencer et al., 2003). In addition to signaling pathways, many major enzymes such as kinases, ATPases, topoisomerase, RNA and DNA polymerase, glutathione S-transferase (GST) and aldose reductase are affected by flavonoids (Middleton, 2000). All these studies indicate that flavonoids may be used for several diseases as a highly potent protective or therapeutic agent.

#### 1.1.1.2 Synthetic antioxidants

Synthetic antioxidants are used in foods and cosmetics industry to prevent food decay and to extend the shelf-life of products (Gamboni et al., 2013; Sanhueza, et al., 2000). Moreover, synthetic antioxidants are used in laboratory antioxidant experiments as standards. BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), tertiary butylhydroxyquinone (TBHQ), and ethoxyquin (EQ) are well-known synthetic antioxidants (Carocho and Ferreira, 2013). BHT, which is a derivative of phenolic compounds and reacts as a synthetic analogue of vitamin E, is added in many food products as a food additive (Halliwell and Gutteride, 1989). BHA is also used as preservative and it was shown that BHA has stronger antioxidant ability than BHT (Karamac and Amarowicz, 1997).

## 1.2 Oxidative Stress

### 1.2.1 Free Radicals and Oxidative Stress

Free radicals are defined as molecule that have unpaired electrons and result in highly reactive, short life and can be potent damaging agent (Halliwell and Gutteride, 1989; Fridovich, 2013). There are two main types of free radicals with respect to their functional group: reactive oxygen species (ROS) (Gerschman et al., 1954) and reactive nitrogen species (RNS) (Rios-Arrabal et al., 2013). Free radical formation occurs by two ways; electron transfer (oxidation or reduction reaction) or production of ionic species by covalent bond cleavage (Table 1.1) (Choudhari et al., 2013).

**Table 1.1** Summaries of chemical reactions in antioxidant defense system. Retrieved from (Rios-Arrabal et al., 2013).

| REACTIVE OXYGEN SPECIES | | REACTIVE NITROGEN SPECIES | |
|---|---|---|---|
| **Sıperoxide Anion** | $O_2 \xrightarrow[\text{oxidase}]{\text{NADPH}} O_2^{-}\bullet$ | **Nitric Oxide** | $\text{L-Arginina} \xrightarrow[\text{NOS}]{\text{L-Citrulina}} NO\bullet$ |
| **Hydrogen Peroxide** | $2O_2^{-}\bullet \xrightarrow[\text{SOD}]{O_2} H_2O_2$ | **Peroxynitrite** | $NO\bullet \xrightarrow{O_2^{-}} \xrightarrow{O_2} ONOO^{-}$ |
| **Hydroxyl Radical** | $H_2O_2 \xrightarrow[\text{Reaction}]{\text{Fenton}} \bullet OH$ | **Dioxide of Nitrogen** | $NO\bullet_2$ |
| **Hydroperoxyl Radical** | $O_2 \xrightarrow{H\bullet} HO_2^{-}\bullet$ | **Anhydride Nitrous** | $NO\bullet \longrightarrow N_2O_3$ |

Oxidative stress is the imbalance between free radicals and antioxidants that may lead to the damage of nucleic acids, proteins and lipids in cells (Sies, 1986). In other words, weakness of antioxidant system or high production of ROS results in oxidative stress through reduction of free radical neutralization capacity (Ray et al., 2012). Extended oxidative stress has been implicated in cardiovascular diseases, carcinogenesis, neurological disorders as well as aging (Lobo et al., 2010).

### 1.2.2 Reactive Oxygen Species

Oxygen is a functional center for reactive oxygen species and well known examples are superoxide ($O_2^-$), hydroxyl radical (•OH), hydrogen peroxide ($H_2O_2$) and singlet oxygen $O_2$ (Olguín and Guzman, 2010). ROS can be classified as radical ROS (superoxide, hydroxyl radical and organic radical) and nonradical ROS (hydrogen peroxide, singlet oxygen and ozone) (Gupta et al., 2012). In normal physiology, ROS is generated as a result of aerobic metabolism and its level is kept under control by antioxidant defense system to maintain oxygen homeostasis (Figure1.5) (Seifried H., 2006).

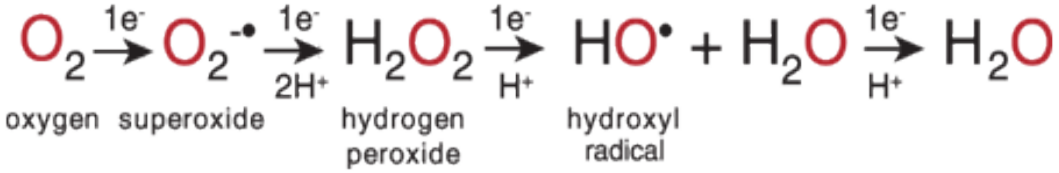


**Figure 1. 5** Sequential reduction of oxygen to water. Retrieved from (Koop, 2006)

Adding one electron to oxygen molecule generates superoxide anion. This reduction reaction of oxygen occurs in the mitochondria as a consequence of electron transport chain mechanism (Fridovich, 2013). Superoxide anion behaves as both oxidizing and reducing agent, which oxidizes ascorbate and reduces cytochrome c. Superoxide dismutase (SOD) enzyme converts superoxide anion to hydrogen peroxide (Cabelli and Bielsk, 1983; Gutowski and Kowalczyk, 2013; Harel and Kanner, 1988).

Hydrogen peroxide belongs to the nonradical class of ROS, which is the end product of superoxide dismutation reaction and the second intermediate during the reduction of oxygen. It has membrane permeability due to lipid solubility characteristic of compound. Although, hydrogen peroxide itself is a stable compound, it produces highly reactive species (hydroxyl radical) through metal catalyzing Fenton and Haber-Weiss reactions (Tauler and Agulio, 2010).

Hyroxyl radical is the most reactive of all the oxygen species with a short half life, and is formed from hydrogen peroxide in the presence of transition metal ion (ferrous iron) as a catalyst by Fenton reaction (Koop, 2006). This very highly reactive species can react with DNA, proteins and cellular components and result in destruction of cell. The damaging reactions of hydroxyl radicals can be prevented by removing the metal ion from the reaction environment. The final product of redox reaction is water with the help of catalase enzymes (Gutowski and Kowalczyk, 2013).

Singlet oxygen is a nonradical reactive oxygen species due to the absence of unpaired electrons leading to increased reactivity of molecule. The end product of reaction between hydrogen peroxide and hypoclorite ion ($OCl^-$**)** is singlet oxygen (Halliwell and Gutteride, 1989).

### 1.2.2.1 Sources of Reactive Oxygen Species

There are both endogenous and exogenous sources for ROS production. Exogenous sources of ROS are water and air pollutants, cigarette smoke, radiation, heavy or transition metals (Cd, Hg, Pb, Fe, As), industrial solvents, singlet oxygen ($O^{-}$), oxides of nitrogen ($NO^{\cdot}$ and $NO^{\cdot}_2$), ionizing radiation, cooking (smoked meat, used oil, fat), drugs and alcohol (Figure 1.6) (Pham-Huy et al., 2008).

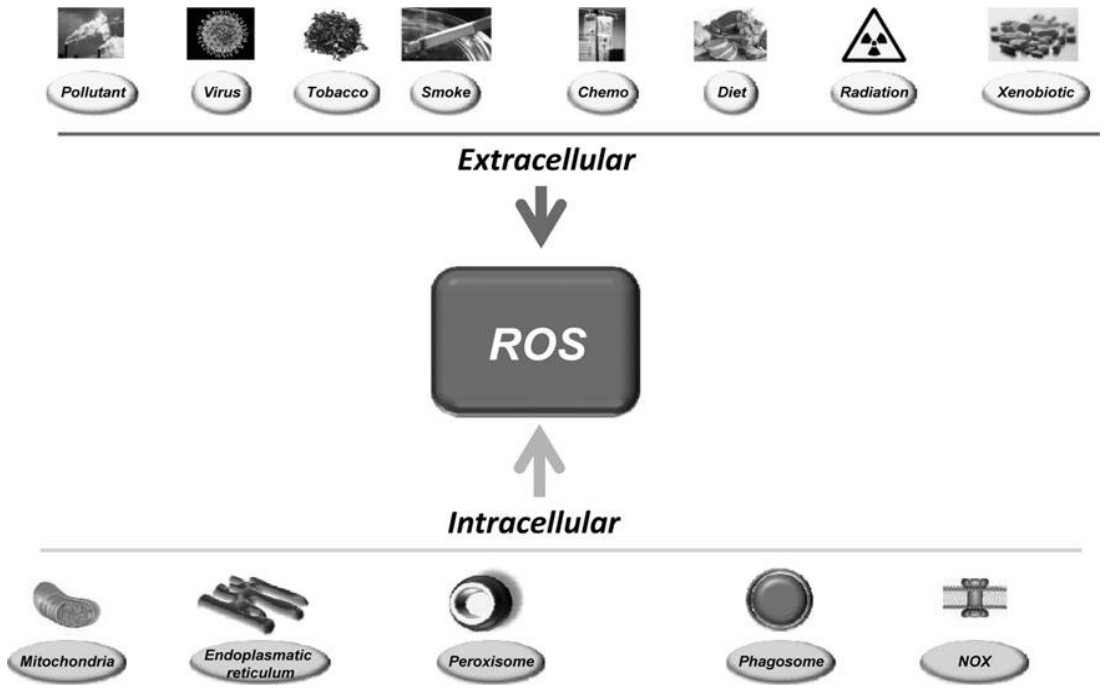


**Figure 1. 6** Exogenous and endogenous sources of ROS production. Retrieved from (Gupta et al., 2012).

ROS can be produced endogenously from the mitochondria, cytochrome P450 metabolism, peroxisomes, inflammatory reactions, arachidonate metabolism pathway, phagocytosis and xanthine oxidase (Lobo et al., 2010). Oxygen consumption to synthesize ATP in mitochondria is essential for aerobic life and leads to ROS generation through electron transport chain reactions. NADPH oxidase enzymes provide reduction of oxygen to superoxide and then to hydrogen peroxide by SOD enzyme (Gupta et al., 2012; Qin and Rodrigues, 2010). Peroxisomes produce free radicals through normal metabolic reactions by NAPH oxidase and xanthine oxidase (XOD) (del Rio et al., 2006). ROS production is elevated through inflammation to kill pathogens and induce inflammatory pathways. Chronic ROS production leads to chronic inflammation (Jabaut and Ckless, 2012).

### 1.2.3 Downstream Targets of ROS

Reactive oxygen species interact with various different molecules that maintain normal physiological redox levels as well as those that are involved in the progression of various diseases. There are several targets of ROS including nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), hypoxia-inducible factor 1-alpha (HIF-1α), signal transducer and activator of transcription 3 (STAT3), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), p53 and others (Figure 1.7) (Gupta et al., 2012) .

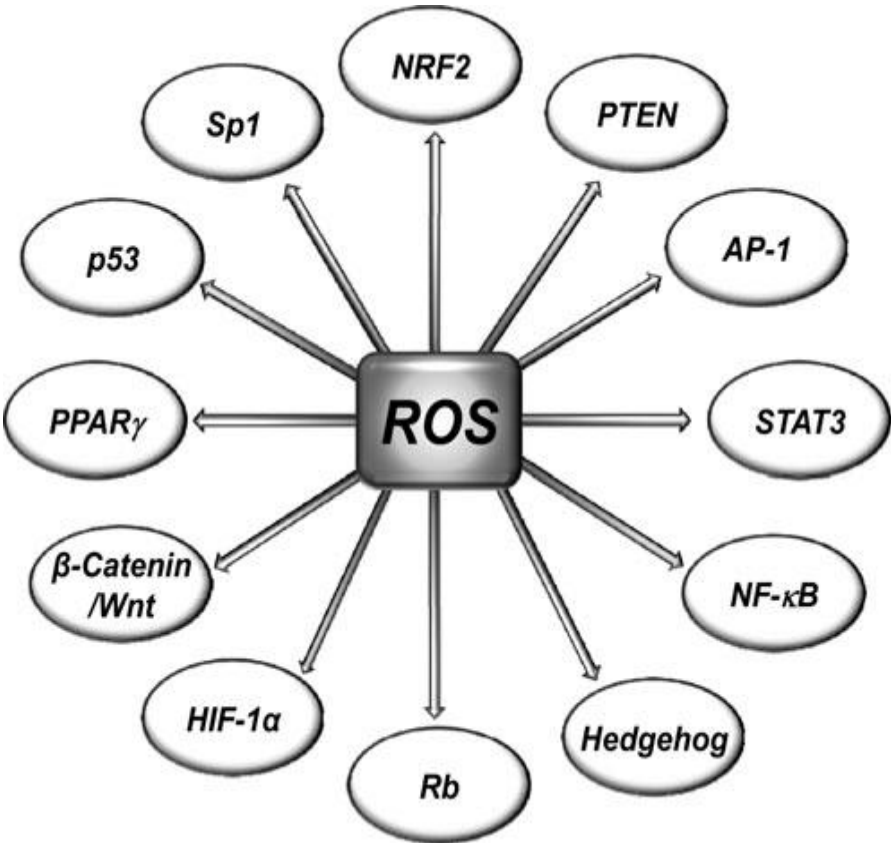


**Figure 1. 7** Targets of Reactive Oxygen Species. Retrieved from (Gupta et al., 2012).

### 1.2.3.1 Mitogen Activated Protein Kinases (MAPKs)

Mitogen activated protein kinase pathway is one of the critical pathways in the regulation of proliferation, apoptosis and differentiation of cells (English and Cobb, 2002; Keyse, 2000; Rho et al., 2011). Based on their sequence homology and nature of activations, these kinases are divided into two classes: growth-factor-activated MAPKs; ERK1 (extracellular-signal-regulated kinase) and ERK2 (MAPK1 and

MAPK2) and stress-activated protein kinases; c-jun amino-terminal kinases (JNK) and p38 (Cohen, 1997).

Phosphorylation site of ERK, JNK and p38 is Thr-Glu-Tyr, Thr-Pro-Tyr and Thr-Gly-Tyr, respectively (Pearson et al., 2001). MAPKs are activated by receptor tyrosine kinase (RTK) and G protein-coupled receptors (GPCRs) that lead to phosphorylation of threonine and tyrosine residues of MAPKs (Keyse, 2000). Upon binding growth factors to RTKs leads to ligand-induced dimerization (Lemmon and Schlessinger, 2010). After dimerization and autophosphorylation of RTKs, GRB2 (adaptor protein with SH2 domain) binds to phosphotyrosine of RTK and provides binding site for SOS protein that is found as a guanine nucleotide exchange protein for activation of membrane bound protein Ras. Ras is inactive when bound to guanosine diphosphate (GDP); replacement of GDP with guanosine triphosphate (GTP) results in activation of the protein (Lodish et al., 2000). Binding of Raf (a serine/threonine kinase) protein to Ras-GTP generates sequential phosphorylation of MAPK kinase kinase (Raf), MAPK kinase (MEK) and MAPK (ERK) through dual specificity (Figure 1.8) (Son et al., 2011).

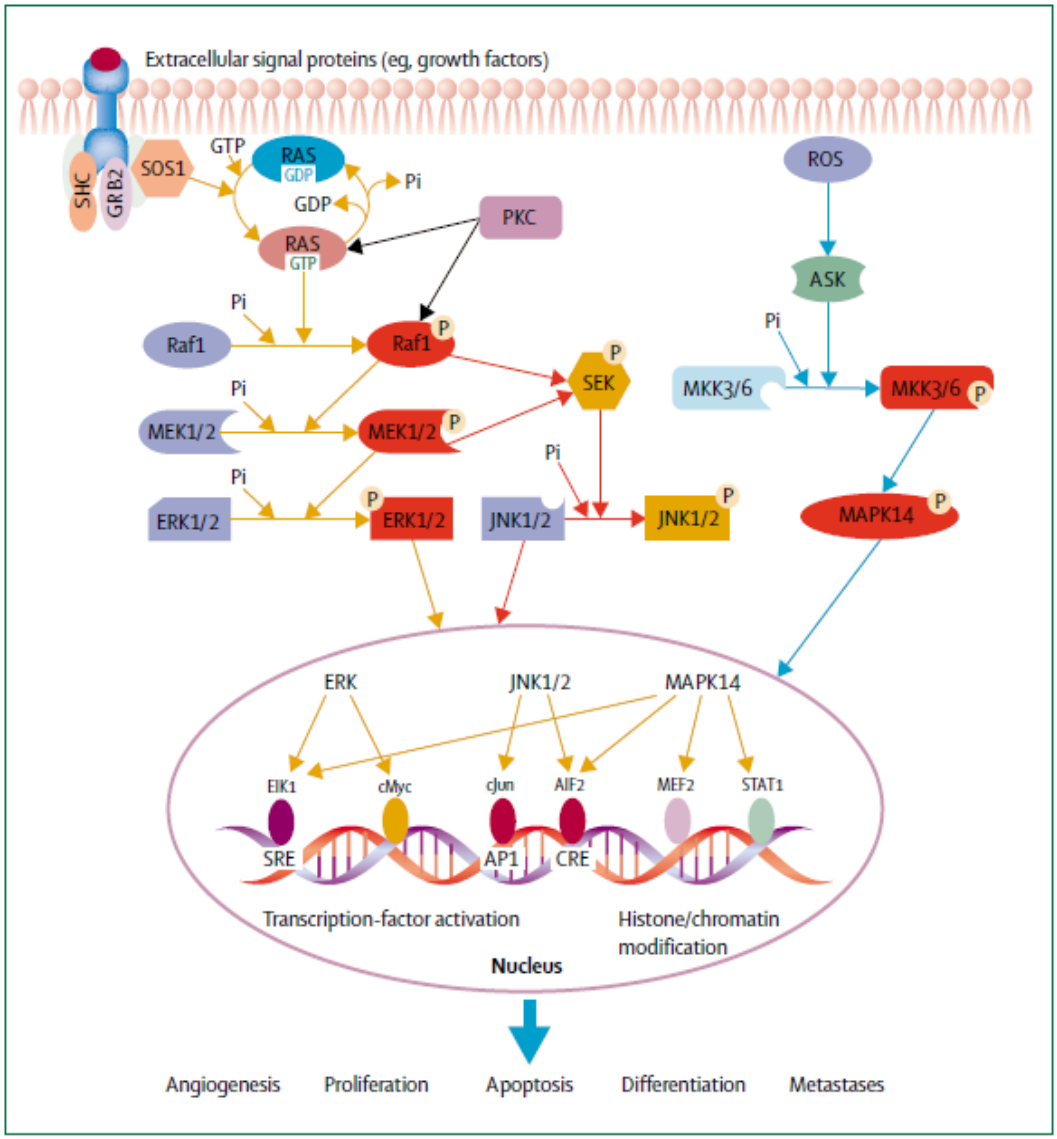


**Figure 1. 8** Schematic representation of MAPK signaling pathway. Retrieved from (Fang and Richardson, 2005)

**Extracellular-signal Regulated Kinase (ERK)**

RAF–MEK–ERK signaling cascade is one of the most potent mitogenic signals in a cell. ERK1/2 (p44/p42 MAPK) are regulated by phosphorylation/dephosphorylation; in other words, phosphorylated ERK1/2 are the active form of kinase that can translocate to the nucleus and induce target gene expression (Takashima and Faller, 2013).

Ras is an oncogene with mutations leading to constitutive activation seen in 36% of colorectal cancers (Andreyev et al., 1998). Constitutive activation of ERK1/2 promotes tumorgenesis through induction of cell proliferation (Fang and Richardson, 2005).

In addition to Ras, mutated BRAF is implicated in kinase activity; moreover, screening results of 43 cell lines indicated that the incidence of BRAF mutation was found as 18% of colorectal cancers. The same research group showed that different mutation of BRAF induced activation of ERK1/2 kinases (Davies et al., 2002).

Targets of ERK1/2 are c-myc, c-Fos, ATF-2 and Elk-1 that contribute to the crucial role of ERK through cell cycle progression, proliferation and survival (Britten, 2013; Zhang and Liu, 2002).

**c-Jun N-terminal Kinase (JNK)**

JNKs are found under stress activated protein kinase (SAPK) family and activated by intracellular and extracellular stress factors such as cytokines, ROS, ER stress, UV exposure and temperature changes (Hayakawa et al., 2012). Apoptosis signal-regulating kinase 1 (ASK1) proteins belong to MAPK kinase kinase (MAP3K) family and are found upstream of JNK and p38 activation cascades (McDonald et al., 2000). JNK shows differing responses depending on the duration of activation. In the

early phase of activation, it promotes cell survival; however, JNK induces pro-apoptotic proteins in late phase of activation such as long term ROS exposure. Moreover, there is a positive feedback loop between ROS and late phase JNK activation (Ventura et al., 2006). Also research on JNK indicates that this family of proteins can act as both tumor suppressor and tumor promoter (Davies and Tournier, 2012).

The major target of JNK is c-jun that forms homodimer or a heterodimer with Fos family or ATF-2 family to create AP-1 (Activator Protein 1). Other downstream targets of JNK are ATF-2 (activating transcription factor 2), Elk-1 and p53 (Ip and Davis, 1998; Zhang and Liu, 2002). JNK pathway mediates cell death and apoptosis, in addition, elevated activity of JNK is found in different cancers (Licato and Brenner, 1998). Nateri et al. used a mouse model with a genetic abrogation of c-Jun N-terminal phosphorylation to investigate effect of c-jun inactivation on carcinogenesis. Results showed reduction of tumor number and size as well as an extension in lifespan (Nateri et al., 2005).

**p38/MAPK**

p38 is a member of stress activated protein kinase (SAPK) family activated by cytokines, growth factors, oxidative stress and DNA damage in the same manner as JNK/SAPK family.

ATF1, ATF2, ATF6, p53, MEF2 or C/EBP beta are generally activated downstream of p38 protein kinases (MAPK14) (Porras A., 2010). p38 is implicated in the regulation of cell-cycle checkpoints ($G_2$/M) and promotion of DNA repair (Cuadrado and Nebreda, 2010; Wood et al., 2009; Zhang and Liu, 2002). p38 activation induces transcription of cytokines by phosphorylation of histone H3 that increases NF-κB recruitment on promoters and also provides chromatin relaxation for DNA repair (Saccani et al., 2002).

Some studies have shown the tumor suppressor characteristics of p38 through cell cycle arrest and induction of apoptosis (Bulavin et al., 2001; Dolado et al., 2007). On the other hand, other studies have indicated that prominent activation of p38 can be observed in hypoxic conditions (Xu et al., 2004) and angiogenesis (Cuenda and Rousseau, 2007). Rajashekhar et al. showed that decreased tumor growth and vessel density were observed in p38 inhibitor treated mice which meant the induction of angiogenesis by p38/MAPK activation (Rajashekhar et al., 2011).

### 1.2.4 ROS and Cancer

Cancer formation is a result of multistep and long-term progress that includes initiation, promotion, progression, invasion and metastasis (Pan et al., 2010). Characteristics for tumor development include sustained proliferative signaling, evasion of apoptosis, sustained growth signals, resistance to growth inhibitory signals, promotion of angiogenesis, and ability to invade and metastasize (Hanahan and Weinberg, 2011). Chronic inflammation and exposure to free radicals (ROS and RNS) promote tumor initiation and progression by DNA damage and mediation of major signaling pathways including MAPK, PI3K and NF-κB (Pan et al., 2010; Ray et al., 2012).

In normal physiological conditions, ROS production is essential for oxygen homeostasis (between oxidants and antioxidants) as a result of aerobic metabolism; however antioxidant defenses may get overwhelmed by excess free radicals and lead to oxidative stress that has been implicated in many different diseases such as diabetes, Alzheimer's disease, atherosclerosis and cancer (Seifried H., 2006; Seifried et al., 2007). ROS promote initiation of carcinogenesis by genetic alteration, damage to biological macromolecules and interference of DNA repair mechanisms (Figure 1.9) (Fortoul et al., 2010) and enhance next steps by maintaining signaling pathways.

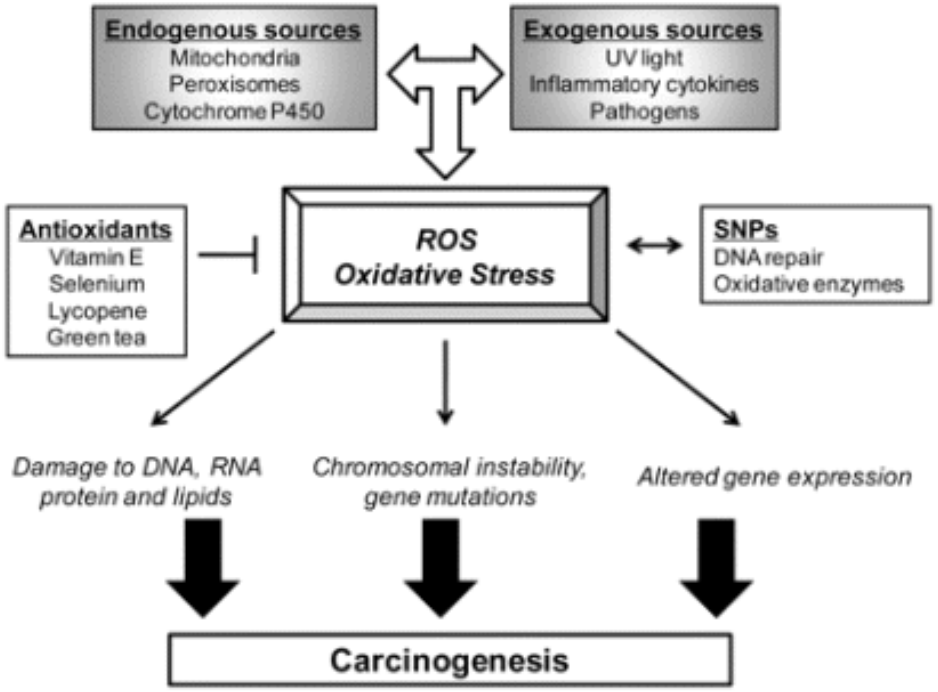


**Figure 1. 9** Role of ROS and antioxidants in carcinogenesis. Retrieved from ( Klaunig et al., 2010).

PI3K/AKT pathway is regulated by ROS products through the oxidation of the tumor suppressor protein PTEN. Protein tyrosine phosphatases have cysteine residues, which are highly reactive. Oxidation of PTEN by $H_2O_2$ results in formation of disulphide bonds between Cys residues (catalytic domain) that inhibits the phosphatase activity of PTEN resulting in the activation of AKT (Dakubo, 2010; Seo et al., 2005). Elevated NF-κB activity is implicated in chronic inflammatory conditions due to high ROS production. This pathway suppresses apoptosis by transcriptionally upregulating anti-apoptotic genes including Bcl-2, Bcl-xL and IAP

(Chaves et al., 2009). Uncoupling protein-2 (UCP2) found in the oxidative defense system, can sense and regulate mitochondrial $O_2^-$ generation. It was shown that $Ucp2^{-/-}$ mice have more aberrant crypt foci formation, increased NF-κB and ERK activity than $Ucp2^{+/+}$ mice (Derdak et al., 2006). Exogenous hydrogen peroxide treatment of SW620 (colon cancer cell line) induced phosphorylation of ERK, JNK and p38. Following the treatment with an antioxidant, the raised MAPK activity was reduced by scavenging action of the antioxidant compound. In addition, the same group reported that $H_2O_2$ treatment leads to enhanced invasion of cancer cells in a Matrigel-coated Boyden chamber invasion assay and increased MMP-7 level (Ho et al., 2011).

Exogenous and endogenous antioxidants have anticarcinogenic effects including inhibition of proliferation by blocking of signaling pathways and strengthening of DNA repair mechanisms (Milbury and Richer, 2008).

### 1.2.5 Reactive Nitrogen Species (RNS)

The functional center of RNS is nitrogen and its reactive forms are nitric oxide (NO•), peroxynitrite ($ONOO^-$), the radical nitrogen dioxide ($NO_2$•) and nitrite ($NO_2^-$) (Olguín and Guzman, 2010). Excess production of reactive nitrogen species leads to nitrosative stress that causes interference of protein structure. The end product of the chemical reaction between superoxide anion and nitric oxide is peroxynitrite, which is a highly reactive compound and is implicated in DNA fragmentation (Valko et al., 2007). Nitric oxide is implicated in many diseases such as malaria, cardiovascular diseases, acute inflammation, neurodegenerative diseases and cancer (Gutowski and Kowalczyk, 2013).

## 1.3 Traditional Medicines

World Health Organization defines traditional medicines as “the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness”. The term is also used for complementary and alternative medicine in some regions (CAM) (World Health Organization, 2001). Traditional medicines have been applied for ages in different societies. The best example of a long standing (over 3000 years) and sophisticated class of traditional medicines that have been extensively used and studied is Traditional Chinese Medicine (TCM) (Wachtel-Galor and Benzie 2011). Considering their multifocal problems, chronic diseases are more complicated than acute diseases. TCM has been described as a polypharmacy where complex mixtures of compounds containing multiple synergistically active ingredients can affect multiple targets simultaneously. Therefore, TCM may be more effective than a single drug against multifocal symptoms (Crow, 2011). As a novel example, ginsenosides (a family of steroid glycosides) that are the primary ingredients of ginseng are not absorbed from gut but many people have positive effects because their gut microbiota provide beneficial remnants from ginsenosides (Crow, 2011).

Ginseng extract is available in the market as a dietary supplement in both tablet and powder form. Dietary supplements constitute major part of complementary and alternative medicine. According to Boon, more than 50% of cancer patients tend to use herbal medicines as a complementary and alternative medicine because dietary supplements (vitamins, minerals and herbals) are thought to be implicated in prevention and treatment of cancer as well as alleviation of serious side effects of modern cancer medicines (Boon and Wong, 2004). Garlic, black cumin, cloves, cinnamon, thyme, allspice, bay leaves, mustard, and rosemary are used commonly in the diet to maintain general health and well-being. Moreover, plant extracts that include phytochemicals such as carotenoids, curcumins, catechins, lignan are consumed for their anti-carcinogenic properties (Lai and Roy, 2004).

Nowadays, use of dietary supplement is very popular for both developing and developed countries (Fong, 2002). According to Euromonitor International Report, the global vitamin and supplement market is worth $68 billion (Report Linker. http://www.reportlinker.com/ci02037/Vitamin-and-Supplement.html).Increased trend of consuming supplements brings about necessity of research into proper and safe dosage, molecular effects and clinical efficacy (Wachtel-Galor and Benzie, 2011).

## 1.4 *SALIX AEGYPTIACA L.*

The common name of *Salix aegyptiaca* is Musk Willow, which is cultivated in Turkey (Southeastern Anatolia), Iran, Turkmenistan and Afghanistan (Table 1.2) (Karimi et al., 2011; Turkish Plant Data Service, 2013).

**Table 1.2** Classification of *Salix aegyptiaca.* Retrieved from (Turkish Plant Data Service, 2013).

| **Rank** | **Scientific Name** |
|---|---|
| Kingdom | Plantae |
| Subkingdom | Tracheobionta |
| Superdivision | Spermatophyta |
| Division | Magnoliophyta |
| Class | Magnoliopsida |
| Subclass | Dilleniidae |
| Order | Salicales |
| Family | Salicaceae |
| Genus | *Salix* L. |
| Species | *Salix aegytiaca* L. |

Over the centuries, Salix extracts, particularly from the bark, have been used to treat different diseases owing to their antioxidant, anti-inflammatory, analgesic, anti-hyperchlosterolemic effects (Asgarpanah, 2012; Enayat and Banerjee, 2009; Mahdi et al., 2006). Willow bark contains many phenolic and flavonoid compounds such as catechin, catechol, salicin, gallic acid, EGCG, vanillin, quercetin and apigenin (Table 1.3) that may give its anti-carcinogenic properties. Enayat et al. have shown that the ethanolic extract from bark (EEB) of *Salix aegyptiaca* did not show any growth inhibitory effect on a non-transformed colon fibroblast cell line (Enayat et al., 2013).

**Table 1.3** Phenolic and flavonoid content of *Salix aegyptiaca* fractions. Retrieved from (Enayat et al., 2013).

Phenolics and Flavonols Present in Ethyl Acetate and Water Fractions of the Extract as Determined by Tandem Mass Spectrometry

| Compounds | Aqueous fraction (ppm) | Ethyl acetate fraction (ppm) |
|---|---|---|
| Catechin | ≤1 | 2311.55 |
| Salicin | 79.59 | 944.7 |
| Catechol | 1.58 | 502.57 |
| Vanillin | ≤1 | 39.9 |
| Galic acid | ≤1 | 30.96 |
| Rutin | 2.32 | 30.04 |
| Syringic | 0.73 | 10.83 |
| EGCG | ≤5 | ≤5 |
| P-coumaric acid | ≤1 | 3.77 |
| Quercetin | ≤1 | 3.41 |
| Apigenin | ≤1 | 0.5 |

EGCG = epigalocatechin gallate. The numbers in parenthesis represent mean and standard deviation of at least 3 independent experiments.

Dietary polyphenolic compounds have ability to inhibit tumorigenesis through the regulation of proliferation, angiogenesis, apoptosis and various mitogenic or tumor suppressive signal transduction pathways. It was reported that EGCG showed reduction of skin, stomach, colon and lung carcinogenesis (Yang et al., 2001) and inhibition on MAPK, NF-κB, AP1 and metalloproteinases (Yao et al., 2011). There are many studies on anti-carcinogenic effect of quercetin. Apigenin and quercetin was shown to be more efficacious than tamoxifen to inhibit the growth of melanoma cells and metastasis (Caltagirone et al., 2000).

Salicin, found in Willow species in high amounts, is the active metabolite of acetyl salicylic acid (ASA, Aspirin ®). Algra reported that long term use of aspirin decreases risk of sporadic colorectal cancer by the inhibition of COX-2 and induction of pro-apoptotic factors (Algra and Rothwell, 2012). Antioxidant characteristics of flavonoids found in Willow species may also contribute to the anti-carcinogenic effect by reduction of cell proliferation, and stimulation of DNA repair system. (Orlikova et al., 2013; Milbury and Richer, 2008; Qin and Sun, 2005).

## 1.5 Aim of the Study

Several research groups have shown that natural compounds have major roles in the treatment of several diseases, especially cancer (Aggarwal and Shishodia, 2006; Boon and Wong, 2004; Wang and Weller, 2006). More than 70% of approved cancer therapeutics comes from natural compounds or their mimetics (Wachtel-Galor and Benzie, 2011). Moreover, free radicals are implicated in the initial step of carcinogenesis through the interference of DNA repair mechanisms, induction of proliferation, damage to macromolcules and gene alterations (Halliwell and Gutteride, 1989; Klaunig et al., 2010; Milbury and Richer, 2008).

Willow extracts have been consumed as an herbal remedy by various different civilizations for several centuries. Hippocrates advised to chew willow bark as a

remedy for fever and pain (Mahdi et al., 2006). Decoctions of *Salix* are still used by Turks and Iranians for depression, neuropathic pain and rheumatoid arthritis (Karimi et al., 2011). Moreover, Willow bark is found in the ministry of health approved medicinal plant list in Turkey (Ministry of health website. http://www.titck.gov.tr/Default.aspx). However, there are limited studies on the antioxidant and anticancer properties of Willow bark as well as the pathways affected by these extracts at the molecular level. Therefore, the aim of this study was to investigate the biochemical pathways that may be implicated in the anti-carcinogenic effect of ethanolic extract from bark of *Salix aegyptiaca*. We propose that the ethanolic extract from bark of *Salix aegyptiaca* may be utilized as chemopreventive agent due to the characteristic of phytochemicals to target several different pathways simultaneously. Therefore, more plant based natural compounds should be analyzed for their toxicity, efficacy and safe dosage to increase conscious consumption.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Chemicals and Cell Culture

U0126 monoethanolate, catechol, gallic acid, catechin, epigallocatechin gallate (EGCG), Folin and Ciocalteu's phenol reagent and DPPH were obtained from Sigma Chemical Co. (Taufkirchen, Germany). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was purchased from Invitrogen (Carlsbad,CA). Annexin V-FITC Apoptosis Detection Kit and Cell proliferation ELISA, Bromodeoxyuridine (BrdU; Colorimetric) kits were purchased from Roche (Mannheim, Germany). Antibodies against ERK1/2 (1:1000), p-ERK1/2 (Thr 202/204/185/187) (1:1000), p-p38(Thr 180/Thr 182) (1:500), α-Tubulin (1:1000), and GAPDH (1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and p38 (1:1000) antibodies were from Cell Signaling (Danvers, MA). JNK (1:500) and p-JNK (1:500) (Thr183/Tyr185) antibodies (Cell Signaling) were obtained from Dr. Uygar H. Tazebay, Bilkent University, Ankara. The dilutions of the antibodies are indicated in parentheses.

HCT-116 human colorectal cancer cell line was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), HT-29 colon cancer cell line and MCF-7 breast cancer cell line were purchased from ŞAP Institute (Ankara, Turkey). PC3 prostate cancer cell line was obtained from Dr. Uddhav

Kelavkar, Mercer University School of Medicine (Savannah, GA). HCT-116, HT-29, MCF-7, and PC3 cells were cultured under ATCC recommended conditions in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 2 mM LGlutamine. The cells were grown at 37$^{o}$C in a humidified incubator with 5% $CO_2$. All cell culture reagents were purchased from Biochrom (Berlin, Germany). For all experiments, the control cells were treated with the vehicle (DMSO).

## 2.2 Plant Selection

Barks of *Salix aegyptiaca* were picked up from Ghaene ghom, Iran according to established guidelines (N'Guessan et al., 2010). The botanical identification was carried out at the herbarium of the Medicinal Plants Research Institute of Shahid Beheshti University of Tehran, Iran. 250 g dried barks of *Musk Willow* were obtained after air drying 1 kg of fresh bark for 1 week at room temperature. The dried bark was chopped into small pieces and powdered in a DeLonghi Coffee Grinder. The powders were stored at 4$^{o}$C in the dark until extraction.

## 2.3 Plant Extraction

Soxhlet extraction method was carried out according to (Rawani et al., 2013) with some modifications. Ethanol was selected as the solvent for extraction according to previous study (Enayat and Banerjee, 2009). Briefly, 16 g dried powdered bark was put in a thimble-holder and solvent flask was filled with 500 ml ethanol to achieve 1 to 30 (w/v) ratio. Extraction process was carried out up to 6 h. Solvent was evaporated by rotary evaporator and then the extract was lyophilized, which was then stored at -20 $^{o}$C until analysis.

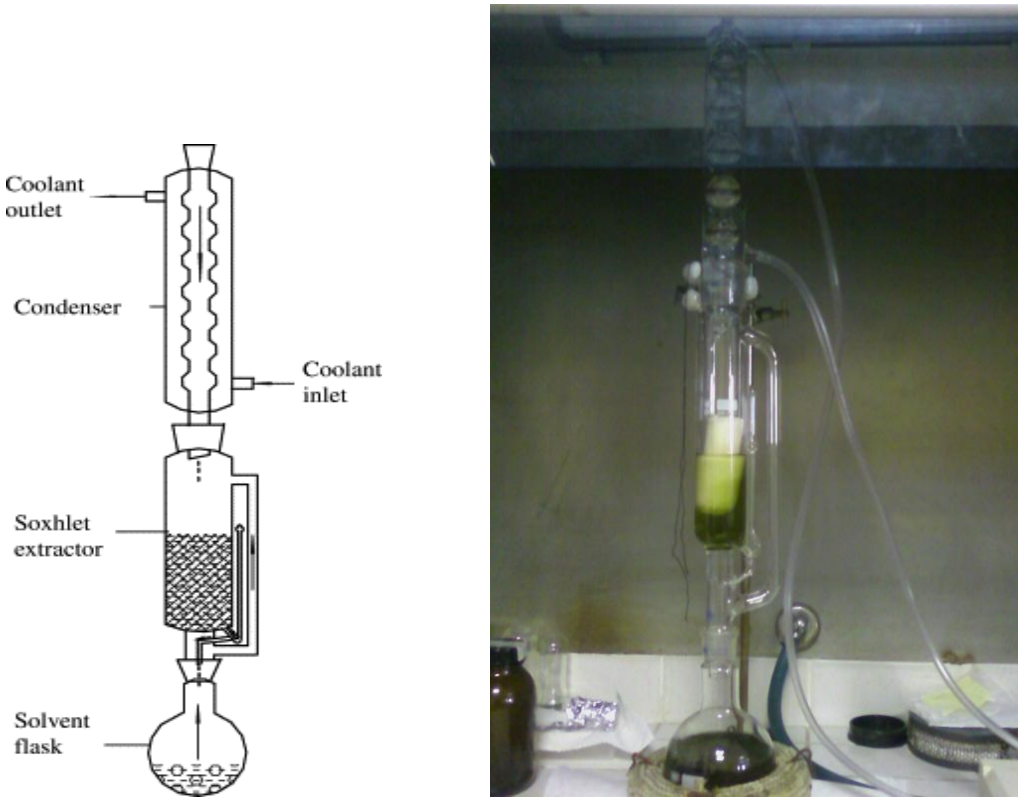


**Figure 2. 1** Schematic representation (Wang and Weller, 2006) and image of our experimental setup

## 2.4 Determination of the Free Radical Scavenging Activity

DPPH (1, 1-diphenyl -2-picryl hydrazyl) assay was carried out to measure the free radical scavenging activity of ethanolic extract of bark according to (Enayat and Banerjee, 2009; Okusa, 2007) protocols. DPPH is a commercially available stable free radical that is widely used to determine the antioxidant capacity of natural compounds. Bleaching of a purple color of the reagent in the presence of a sample indicates its antioxidant capacity which is measured at 515 nm. An $IC_{50}$ value can be

obtained to indicate the sample concentration needed to reduce the absorbance initial DPPH concentration by 50% (Huang et al., 2005). The same amount of methanol and DPPH was used as blank sample as a control (Sini et al., 2010). Calculation of free radical scavenging activity was done by following formula:

$$\% \text{ of DPPH Radical Scavenging Activity} = \left(\frac{\text{Absorbance of DPPH-Absorbance of Sample}}{\text{Absorbance of DPPH}}\right) \times 100$$

Briefly, serial dilutions were prepared from 10 mg/ml stock solution in methanol to achieve accurate concentrations in 96-well plates. Ethanolic extract of bark was then prepared in a concentrations range of 5 µg/ml to 90 µg/ml. 325 µl of freshly prepared 0.005% DPPH solution was added into the well followed by 25 µl of the EEB samples from each dilutions. The plates were incubated in the dark at room temperature for 30 min and 60 min and the absorbance was measured at 490 nm (Hwang et al., 2001) in a BioRad 680 microplate reader (BioRad, USA). The $IC_{50}$ value was calculated from equation of % of DPPH radical scavenging activity vs. concentration (Appendix A). Each assay was carried out 6 times.

**2.5 Determination of Total Phenolic Content**

Folin Ciocalteu (FC) method was used to measure phenolic content of bark according to the protocol of Enayat and Banerjee (2009). The FC assay is based on electron transfer in alkali medium that causes oxidation reduction reaction between phenols and phosphomolybdic/phosphotungstic acid complexes. Therefore, phenolic compounds are reduced and products detected at 765 nm (Ainsworth and Gillespie, 2007). Gallic acid was used as a the standard reference phenolic compound (Singleton et al., 1999).

Briefly, stock solution of gallic acid was prepared as 0.05 g in 1 ml ethanol and diluted to 10 ml with $dH_2O$. Different concentrations of gallic acid (0 to 500 mg/L) were used to generate the standard curve. 1 mg/ml EEB stock solution was used to get 10 µg/ml final concentrations. 3.5 µl extract, standards and water (blank) were placed in 276.5 µl $dH_2O$ and 17.5 µl from 2N FC reagent was added into each well. After incubation for 8 min at 25$^oC$, 52.5 µl from 7% $Na_2CO_3$ was added in each well. Samples were completed to 350 µl with 276.5 µl $dH_2O$. All wells were mixed with pipetting and at the end of 2 h incubation in dark; the absorbance was measured at 765 nm.

A standard curve was created from different gallic acid concentrations for the calculation of the total phenol content of EEB (Appendix B). Results were expressed as mg of gallic acid equivalents (GAE) /100 g of dried sample from following formula;

$$T = C \times V / M \times 100$$

Here, T is the total phenolic content in mg/100 g of the extracts as GAE (gallic acid equivalent), C is the concentration of gallic acid established from the calibration curve in mg/ml, V is the volume of the extract solution in ml and M is the weight of the extract in g (Abdelhady et al., 2011). Each assay was carried out 4 times.

### 2.6 Determination of Total Flavonoid Content

The aluminum chloride colorimetric method was used to measure total flavonoid content of EEB according to Enayat and Banerjee (2009). This assay based on the formation of a stable complex between aluminum chloride and the orthodihydroxyl groups in the A- or B-ring of flavonoids (Marby et al., 1970).

Briefly, different concentrations of (-)-epigallocatechin gallate (EGCG) (0 to 500 mg/L) were used to generate a standard curve. 35 µl from EEB (1mg/ml) stock, (-)-epigallocatechin gallate (EGCG) standards and $dH_2O$ as a blank were mixed with 140 µl $dH_2O$ and 10.5 µl of 5% sodium nitrite ($NaNO_2$). After 5 min incubation, 10.5 µl of 10% $AlCl_3$ was added into each well, and then incubated 6 min at RT. Then 70 µl of 1 N NaOH was added and the total volume was completed to 350 µl. All wells were mixed with pipetting and the absorbance was measured at 490 nm.

A standard curve was created from different (-)-epigallocatechin gallate (EGCG) as reference which was used in calculation of total flavonoid content of EEB (Appendix C). Results were expressed as mg of (-)-epigallocatechin gallate (EGCG) equivalents /100 g of dried sample by using same formula (Section 2.5). Each assay was carried out 6 times.

## 2.7 Proliferation Assays

### 2.7.1 MTT Assay

Effect of EEB and some pure compounds on cell survival was determined by MTT viability assay according to manufacturer's instructions (Invitrogen). This colorimetric assay is based on reduction of water soluble MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to insoluble formazan by viable cells. Color change from yellow to purple is measured at 570 nm in a microplate reader and allows for the quantification of metabolically active cells.

Briefly, the cells ($10^4$ cells/well) were seeded in 96-well plates and after 24 h, cells were treated with different concentrations of EEB (0 to 500 µg/ml) for 48 h. 12 mM MTT was prepared freshly and protected from light. After 48 h incubation, the medium was removed and 100 µl phenol free medium with 10 µl MTT solution was added to each well. SDS-HCI solution was prepared as 1 g SDS in 10 ml 0.01 HCI

by using a bath sonicator (Bandelin Sonorex, Germany). The plate was incubated at 37$^{o}$C for 4 h and then 100 µl/well of 10% SDS-HCI was added to each well. After 18 h incubation at 37 $^{o}$C in a humidified incubator with 5% $CO_2$, absorbance was measured at 570 in a BioRad 680 microplate reader (BioRad, USA). A well which contained medium and MTT labeling solution but no cells was used as a blank. Each assay was carried out 3 times with 5 replicates. Calculations of the growth inhibitory percentage (GI %) for each concentration was done by following formula:

$$\frac{\text{Mean Absorbance of Treated Cells}}{\text{Mean Absorbance of Untreated Cells}} \text{ X } 100$$

### 2.7.2 BrdU Incorporation Assay

BrdU incorporation assay was carried out to determine effect of EEB on cell proliferation according to manufacturer's instructions. BrdU (5-bromo-2'-deoxyuridine) is a thymidine analogue, which incorporates into DNA at the S phase of the cell cycle. Cell division can be detected by the colorimetric BrdU proliferation assay in a microplate reader at 370 nm.

Briefly, the cells ($10^4$ cells/well) were seeded in 96-well plates and after 24 h; cells were treated with different concentrations of EEB (0 to 500 µg/ml) for 48 h. The medium was removed, and 90 µl of fresh medium and 10 µl of BrdU labeling solution were added in each well. The cells are incubated for 2 h during which BrdU is incorporated into DNA. The labeling medium was removed and the cells were fixed with 200 µl/well of FixDenat solution for 30 min at 25 $^{o}$C. Following this, 100 µl/well of anti-BrdU-POD antibody was added and incubated 90 min at 25 $^{o}$C to form immunocomplexes with BrdU. The anti-BrdU-POD solution was removed by extensive washing with 200 µl/well of the washing solution for 3 times. Before measurement, 100 µl/well of substrate solution (Tetramethylbenzidine) was added

into each well and color development occurred within 5-15 min. The absorbance was measured at 370 nm and calculations of the growth inhibitory percentage (GI %) were done by same formula as the MTT assay. Some wells were used for blank and background controls as shown in the table below.

**Table 2.1** Contents of blank and control samples

| **Well Contents** | **Blank** | **Background Control** |
|---|---|---|
| Culture Medium | 100 µl | - |
| Cells | - | 100 µl |
| BrdU Labeling Solution | 10 µl | - |
| Anti-BrdU-POD Solution | 100 µl | 100 µl |

## 2.8 Protein Isolation

HCT-116, HT-29, PC3 and MCF-7 cells ($50x10^4$ cells/well) were seeded in 6-well plates. When the cells reached 60-70% confluence, the cells were treated with EEB at their respective $IC_{50}$ values for 48 h. Control wells contained the appropriate medium at the same volume. At the end of incubation, total proteins were isolated with freshly prepared lysis buffer containing Mammalian Protein Extraction Reagent-M-PER (Pierce, USA), protease inhibitors cocktail and phosphatase

inhibitors (Roche, Germany) according to the manufacturer's instructions. Protein quatification were carried out by Coomassie Plus Protein Assay Reagent (Thermo, USA) according to the manufacturer's instructions and absorbance was measured at 595 nm. Standard curve was prepared with different concentrations of BSA to quantify the protein amount in the lysates (Appendix E).

### 2.9 Western Blotting Assay

Total cell lysates (50 μg) were loaded onto 10% polyacrylamide gel along with a PageRuler prestained protein ladder (10 to 250 kDa) (Thermo Scientific, USA). After electrophoresis, the proteins were transferred from the gel to PVDF membranes for 75 min at 4 $^{o}$C and 100 V. 5% skim milk in PBS-T was used for blocking of membranes for the detection of nonphosphorylated proteins whereas 5% BSA in TBS-T was used for blocking membranes for phosphorylated proteins. After blocking for 1 h at room temperature or overnight at 4$^{o}$C, the membrane was probed with the appropriate primary antibodies diluted either in skim milk or BSA. The antibodies used in this study include ERK1/2 (1:1000), p-ERK1/2 (Thr 202/204/185/187) (1:1000), p38 (1:1000), p-p38(Thr 180/Thr 182) (1:500), JNK (1:500) and p-JNK(Thr183/Tyr185) (1:500), α-Tubulin (1:1000), and GAPDH (1:1000 ). After overnight incubation at 4 $^{o}$C, the membrane was washed 3 times for 10 min with a washing buffer. The membrane was then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:2000 dilutions) and then washed 3 times for 10 min with a washing buffer. The Enhanced chemiluminescence kit (ECL Plus; Pierce) was used to detect bands from immunoblot by exposing to an X-Ray film.

Loading controls was used to confirm that the same amount of protein was loaded in gel and transferred on membrane for all lanes. Before selection of the loading control, expression level of protein were checked for different cell lines and treatments. Housekeeping genes are used commonly as loading control due to their

constant expression levels. GAPDH and α-Tubulin were used as loading control to ensure equal loading. Before re-probing, mild stripping buffer was applied to the membrane at 50 $^{o}$C for 5 min and then washing and blocking steps were carried out with same blot.

### 2.10 Annexin V Assay

Effect of EEB on apoptosis was evaluated by the Annexin-V-FLUOS Staining Kit (Roche, Germany) according to the manufacturer's instructions. When a cell enters process of programmed cell death, phosphatidylserine (PS) switch to the outer leaflet of the plasma membrane from the inner leaflet (Taylor et al., 2008). Annexin V proteins bind to PS and this complex can be detected easily with a flow cytometer.

Briefly, HCT-116 cells were treated with EEB for 48 h in 6-well plates. As a positive control, the cells were treated with 5 mM NaBt for 48 h. At the end of incubation, $10^6$ cells/well were washed with PBS and centrifuged at 200 x g for 5 min. In the mean time, Annexin-V-FLUOS labeling solution was prepared with incubation buffer, Annexin-V labeling reagent and propidium iodide (PI). After centrifugation, the pellet was resuspended in 100 µl of labeling solution and incubated for 10-15 min at room temperature. Samples were analyzed by fluorescence-activated cell sorter analysis (FACS) using a FACScan flow cytometer (Becton–Dickinson, NJ, USA). The results were expressed as a percentage for each phase. Low Annexin-V and low PI staining indicated viable cells, high Annexin-V and low PI staining indicated apoptotic cells and low Annexin-V and high PI staining indicated necrotic cells. The FACScan experiments were carried out at the Department of Molecular Biology and Genetics, Bilkent University.

## 2.11 Statistical Analysis

GraphPad Prism 5 software package was used for data analysis and graphing. Three independent biological replicates were carried out with at least 3 technical replicates. Results are represented as the mean ± S.D. (standard deviation). One-way ANOVA, with post hoc Tukey's multiple comparison tests were carried out to determine significancy of results. A probability (p) value $< 0.05$ was considered as statistically significant.

# CHAPTER 3

# RESULTS

## 3.1 Antioxidant Capacity of the Ethanolic Extract of Bark (EEB) and its Fractions

Free radicals can be counted as one of the major initiators of cancer. Harmful effects of ROS/RNS are tolerated by antioxidant compounds through scavenging and detoxifying of free radicals. Therefore, a high antioxidant capacity can result in more protective effect on diseases. The ethanolic extract of bark (EEB) and its two sub-fractions (ethyl acetate, aqueous) were obtained from the bark of *Salix aegyptiaca* and their free radical scavenging activity, total phenolic and flavonoid contents were evaluated in order to determine their antioxidant capacities.

### 3.1.1 Determination of Free Radical Scavenging Activity by DPPH Assay

Free radical scavenging activity of EEB and fractions were determined by DPPH (1, 1-diphenyl -2-picryl hydrazyl) assay as described in Materials and Methods (Section 2.4). $IC_{50}$ values for each sample were calculated from the percentage of DPPH free radical scavenging activity vs. concentrations (μg/ml) graph (Appendix A). Results showed free radical scavenging activity of the EEB whose $IC_{50}$ value at 30 min and

60 min were calculated as 23 ± 1.5 and 21 ± 1 μg/ml, respectively. $IC_{50}$ values of ethyl acetate, aqueous fractions, n-hexane fractions and n-butanol fractions were 13 ± 1 μg/ml (at 30 min) and 11 ± 1 μg/ml (at 60 min), 38 ± 2 μg/ml (at 30 min) and 33 ± 2 μg/ml (at 60 min), 56 ± 3 μg/ml (at 30 min) and 47 ± 2 μg/ml (at 60 min), 52 ± 2 μg/ml (at 30 min) and 45 ± 1 μg/ml (at 60 min), respectively. Figure 3.1 indicated that the highest antioxidant activity was found in ethyl acetate fraction and the lowest antioxidant activity belonged to n-hexane fraction. EEB, ethyl acetate and aqueous fractions were used in next further experiments due to their low $IC_{50}$ values. The data are displayed with mean ± standard deviation of four replicates.

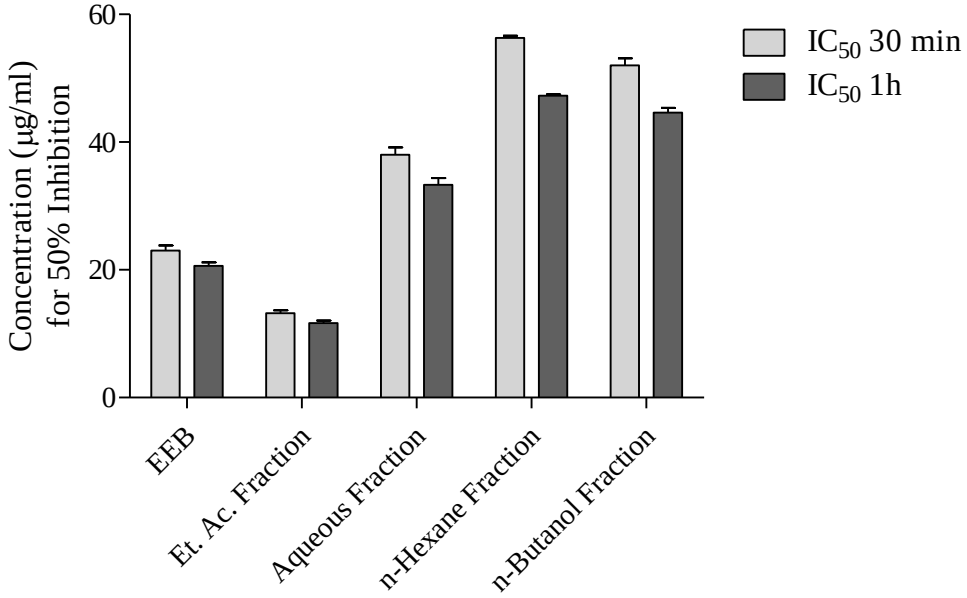


**Figure 3. 1 DPPH radical scavenging activity assay for EEB, ethyl acetate, aqueous, n-hexane and n-butanol fractions at different time points (30 min and 1 h).** Concentration (μg/ml) for 50% DPPH quenching was calculated by using an equation which was generated from free radical scavenging activity (%) vs concentration graph. The lower $IC_{50}$ values indicate better radical quenching activity. The data are displayed with mean ± standard deviation of four replicates.

### 3.1.2 Determination of Total Phenolic Content by Folin-Ciocalteu Method

The total phenolic content was determined by the Folin-Ciocalteu method as described in Materials and Methods (Section 2.2). Gallic acid was used as standard and new standard curves were prepared for each assay. The phenolic content was reported as gallic acid equivalents, which was calculated for each sample from the standard curve (Appendix B). Figure 3.2 shows that the highest phenolic content was obtained in the ethyl acetate fraction (29.6 ± 2 mg GAE/100 g dried weight). Total phenolic content of EEB and aqueous fraction were 25 ± 1.5 and 7.8 ± 1 mg GAE/100 g dried weight, respectively. The data are displayed with mean ± standard deviation of four replicates.

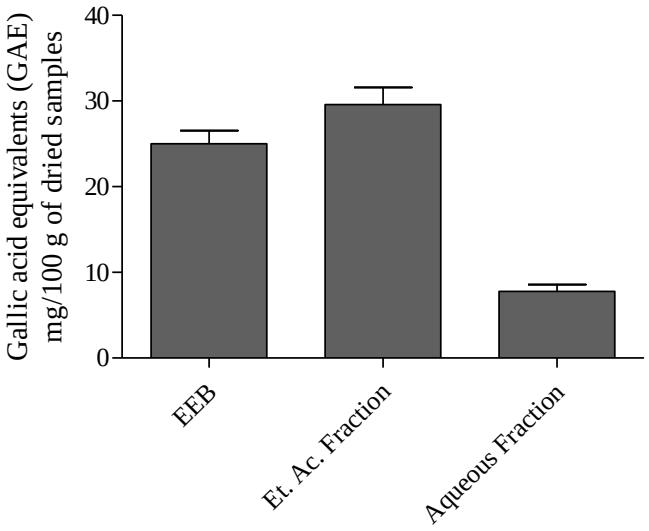


**Figure 3. 2 Total phenolic content of EEB, ethyl acetate and aqueous fractions were determined by Folin Ciocalteu method**. Gallic acid was used as a standard and its equivalence was calculated from absorbance vs gallic acid concentration graph. Higher total phenolic content was seen in EEB and the ethyl acetate fraction when compared to the aqueous fraction. The data are displayed as mean ± standard deviation of four replicates.

### 3.1.3 Determination of Total Flavonoid Content by Aluminum Chloride Colorimetric Method

Total flavonoid content was determined by Aluminum Chloride Colorimetric Method as described in Materials and Methods (Section 2.6). (-)-epigallocatechin gallate (EGCG) was used as standard and new standard curves were prepared for each assay. The total flavonoid content was calculated as EGCG equivalents and was calculated for each sample from equation of standard curve (Appendix C). Figure 3.3 shows that the highest flavonoid content was determined in EEB (47.5 ± 2 mg of EGCG

equivalent/100 g dried weight). Flavonoid content of ethyl acetate and aqueous fractions were 29.5 ± 1.5 and 10.4 ± 1 mg of EGCG equivalent/100 g dried weight, respectively. The data are displayed as mean ± standard deviation of four replicates.

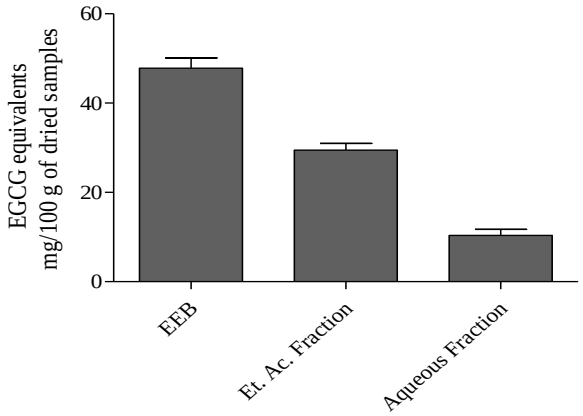


**Figure 3. 3 Total flavonoid content of EEB, ethyl acetate and aqueous fractions were determined by aluminum chloride colorimetric method.** EGCG was used as a standard compound and its equivalence was calculated from absorbance vs EGCG concentration graph. The highest total flavonoid levels were seen in EEB followed by the ethyl acetate and the aqueous fractions. The data are displayed as mean ± standard deviation of four replicates.

## 3.2 Effect of the EEB and its Fractions on Cellular Proliferation

Several antioxidant compounds show inhibitory effects on cellular proliferation due to their high content of both flavonoids and phenolic compounds (Kampa et al., 2000; Kuntz et al., 1999). We therefore next investigated the effects of these extracts on cellular proliferation by MTT and BrdU incorporation assays in different cancer cell lines.

### 3.2.1 BrdU Cell Proliferation Assay

Investigation of antiproliferative effect of EEB and its active fractions was carried out with the BrdU incorporation assay as described in Materials and Methods (Section 2.7.2). 24 h after plating, HT-29 cells were treated with different concentrations (0 to 500 µg/ml) of EEB, ethyl acetate and aqueous fractions for 48 h. Concentrations that led to 50% of loss in proliferation were calculated for EEB and ethyl acetate fractions as 295 ± 19 µg/ml and 227 ± 25 µg/ml, respectively (Figure 3.4). Interestingly, for the HT-29 cell line, the aqueous fractions did not show any significant inhibition on proliferation within the same range of concentrations used. Additionally, the antiproliferative effects of EEB and its fractions in HT-29 cells were assayed with the MTT assay and $IC_{50}$ values were found to be similar to the BrdU results (Enayat et al., 2013).The data are displayed with mean ± standard deviation of three replicates.

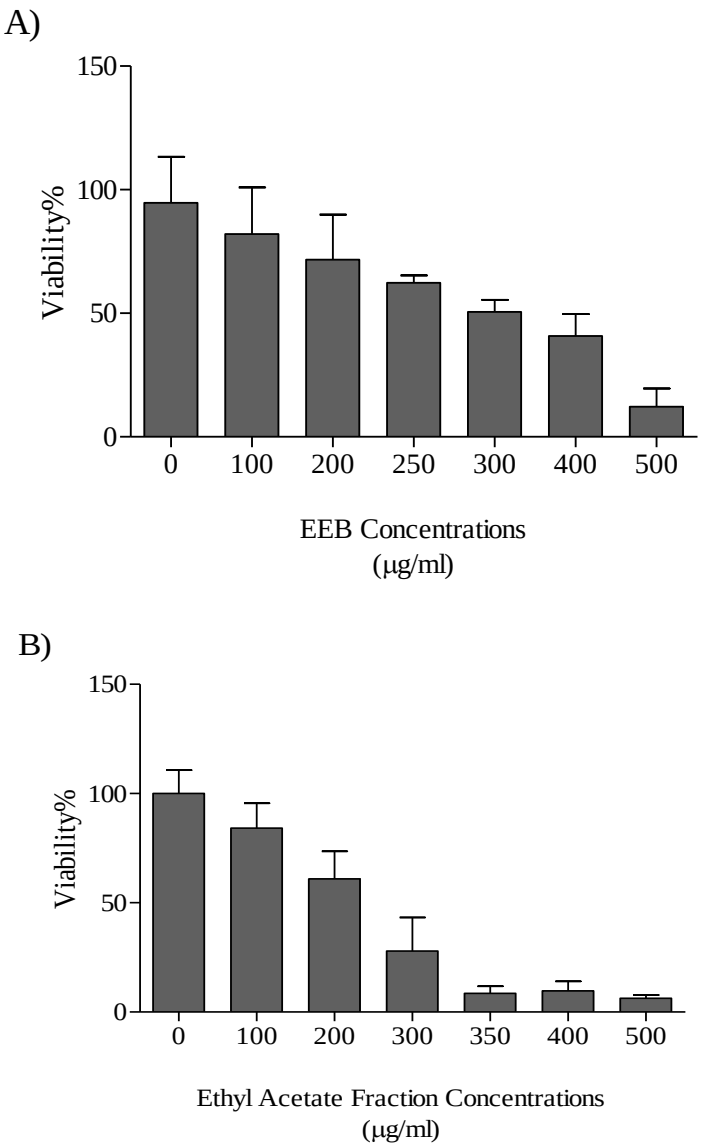


**Figure 3. 4 Growth inhibitory effect of (A) EEB, (B) ethyl acetate fraction and (C) aqueous fraction were carried out by BrdU incorporation assay for 48 h treatment on HT-29 colon cancer cell line**. $10^4$ cells/well were seeded into 96 well plates in complete McCoy's 5A medium. After 24 h seeding, cells were treated with (0-500 μg/ml) EEB, ethyl acetate fraction and aqueous fraction for 48 h. Their $IC_{50}$ values were calculated from viability (%) vs concentration graph. The data are displayed with mean ± standard deviation of three replicates (continued on the next page).

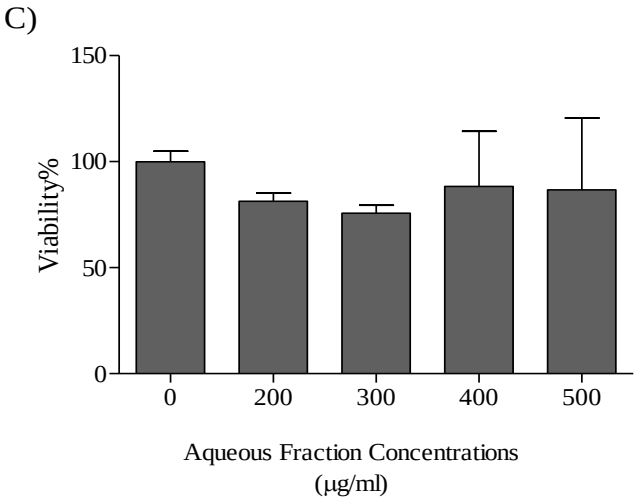


**Figure 3. 4** Continued

### 3.2.2 MTT Cell Proliferation Assay

Effect of EEB and its fractions on proliferation was also determined by MTT cell proliferation assay as described in Materials and Methods (Section 2.7.1). 24 h after plating the cells, HCT-116 cells were treated with different concentrations (0 to 500 µg/ml) of EEB, ethyl acetate and aqueous fractions for 48 h. Concentrations that led to 50% of growth inhibition were calculated for EEB, ethyl acetate and aqueous fractions as 275 ± 9 µg/ml, 192 ± 5 µg/ml and 190 ± 5 µg/ml, respectively (Figure 3.5). Also antiproliferative effects of EEB and its fractions in HCT-116 cells were explored by BrdU assay and $IC_{50}$ values were detected similar with MTT results (Enayat et al., 2013). We have observed that the n-hexane and n-butanol fractions did not show any inhibitory effect on proliferation (Enayat et al., 2013); therefore, we selected the ethyl acetate and aqueous fractions for further experiments.

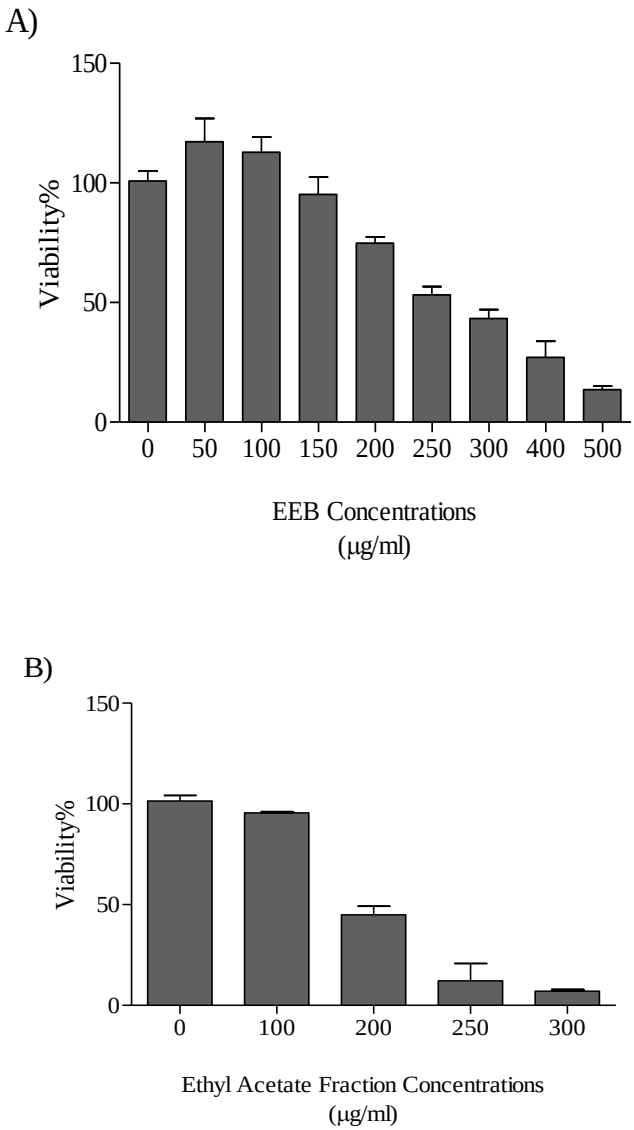


**Figure 3. 5 Growth inhibitory effect of (A) EEB, (B) ethyl acetate fraction and (C) aqueous fraction were carried out after 48 h treatment of the HCT-116 colon cancer cell line with an MTT assay**. $10^4$ cells/well were seeded into 96 well plates in complete McCoy's 5A medium. After 24 h seeding, cells were treated with (0-500 µg/ml) EEB, ethyl acetate fraction and aqueous fraction for 48 h. Their $IC_{50}$ values were calculated from viability (%) vs concentration graph. The data are displayed with mean ± standard deviation of four replicates (continued on the next page).

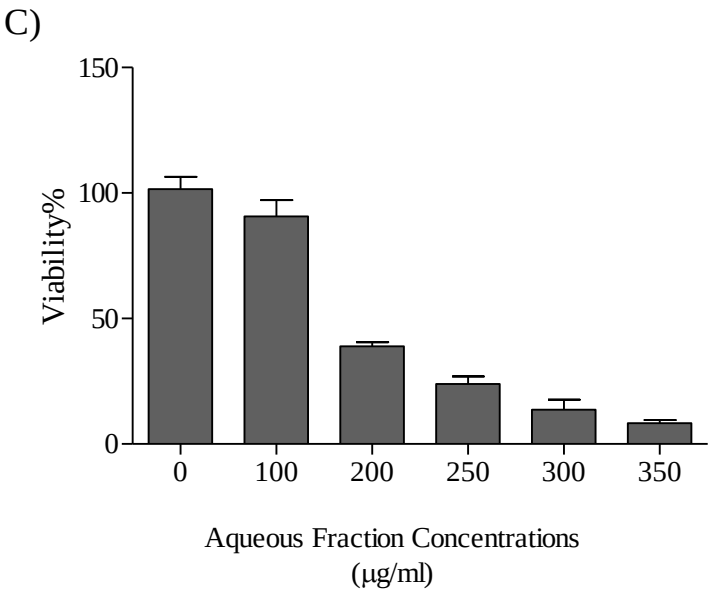


**Figure 3. 5** Continued

Having observed the inhibitory effect of the ethyl acetate and aqueous fractions of EEB on cellular proliferation, tandem Mass Spectrometry (MS-MS) analysis was carried out to determine their active compounds that might result in antiproliferative effect. Predominant compounds that were detected include 2311.55 ppm of catechin, 944.7 ppm of salicin, and 502.57 ppm of catechol in 50 mg/ml ethyl acetate fraction solution and 79.59 ppm of salicin in same concentration of aqueous fraction solution (Enayat et al., 2013).

The concentrations of catechin, catechol and salicin compounds available in 300 µg/ml (maximum observed $IC_{50}$ value for HCT-116 cell line) of EEB were calculated from MS data to be 13.83 µg/ml, 3 µg/ml, and 5.67 µg/ml, respectively. MTT assays were carried out with different concentrations of pure commercially available catechin (0-200 µg/ml), catechol (0-10 µg/ml) and salicin (0-400 µg/ml) compounds for 48 h treatment to determine their effects on proliferation in HCT-116 cells. Figure 3.6 shows that single treatment of pure compounds did not show any

significant antiproliferative effect but when two of them (catechin and catechol) were combined and applied to the cells for 48 h, a strong inhibitory effect on proliferation was observed ($**p < 0.01$ and $***p < 0.0001$ ). Therefore, the combination of phytochemicals showed a significantly greater efficacy on proliferation at low dose compared to single compound treatment. As a result, all future studies were conducted with EEB and its fractions rather than a single pure compound.

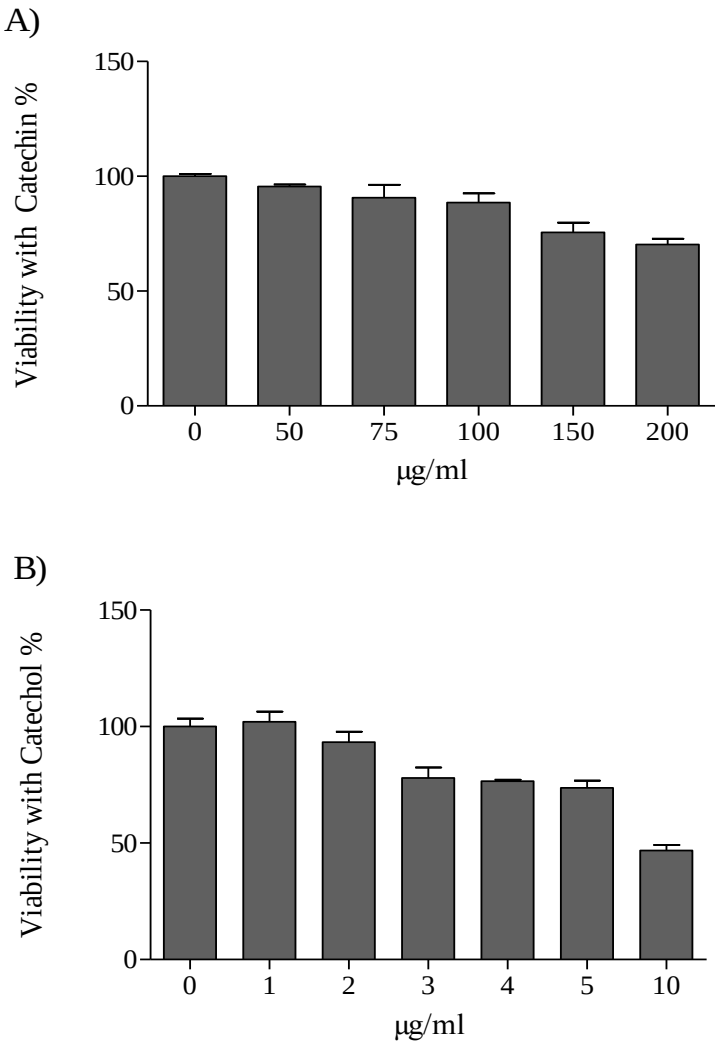


**Figure 3. 6 Effects of pure compounds on proliferation of HCT-116 cells were measured following 48 h treatment of (A) catechin, (B) catechol, (C) salicin and (D) combination of catechin and catechol by MTT assay.** $10^4$ cells/well were seeded into 96 well plates in complete McCoy's 5A medium. After 24 h seeding, cells were treated with different concentration of catechin, catechol and salicin for 48 h. The data are displayed as mean ± standard deviation of at least three replicates.Concentrations were selected according to their equal amounts in 300 µg/ml EEB. Significant differences were obtained in comparison to the control untreated cells and combination (catechin and catechol) treated cells at $**p < 0.01$ and $***p < 0.0001$ by one-way Anova with post hoc Tukey's multiple comparison test (continued on the next page).

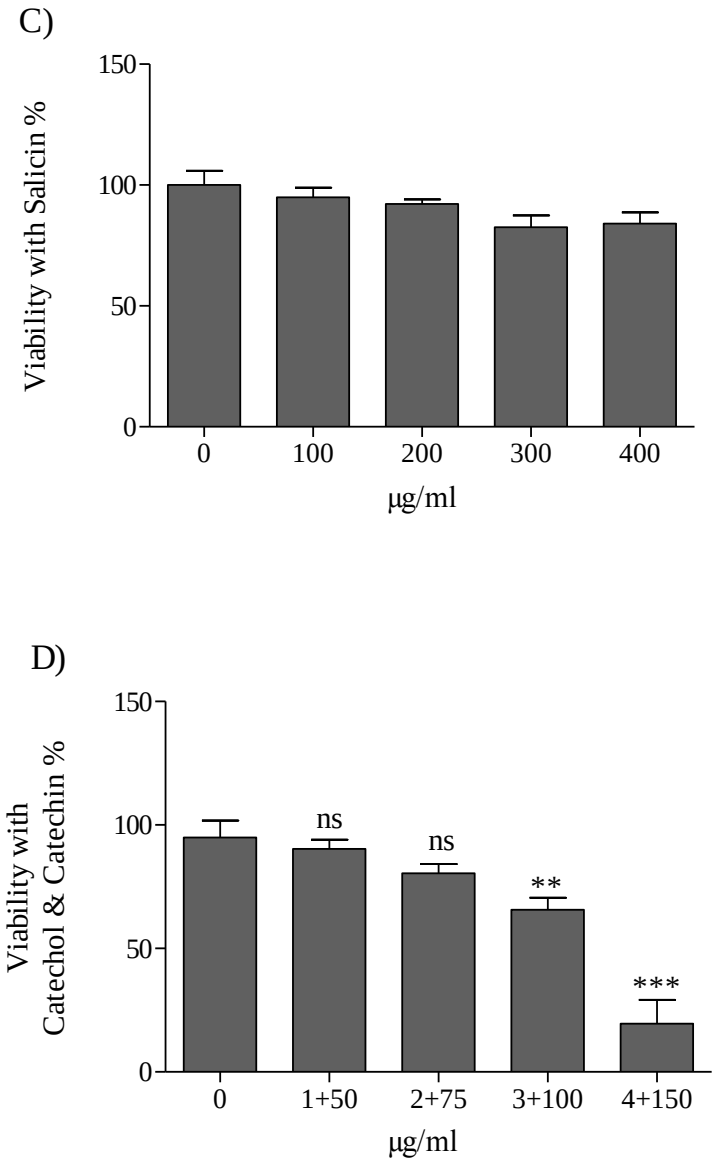


**Figure 3. 6** Continued

### 3.3 Effect of the Active Fractions on Apoptosis

We have previously shown that treatment of colon cancer cell lines with EEB resulted in the induction of apoptosis after 48 h of treatment (Enayat et al., 2013). To determine whether the ethyl acetate and aqueous fractions also led to the induction of apoptosis in HCT-116 cells, Annexin V staining followed by flow cytometry was carried out after 48 h treatment. Figure 3.7 indicates that both ethyl acetate and aqueous fractions significantly increased the apoptosis of these cells compared to vehicle treated control cells $*p < 0.05$ and *** $p < 0.0001$. The apoptotic cell populations detected in untreated cells, 200 μg/ml of ethyl acetate fraction treated cells and 350 μg/ml of aqueous fraction treated cells were 16%, 35% and 37%, respectively. Treatment with 5 mM sodium butyrate (NaBt) for 48 h was used as positive control. The assay was repeated three times and a representative figure is shown in Figure 3.7.

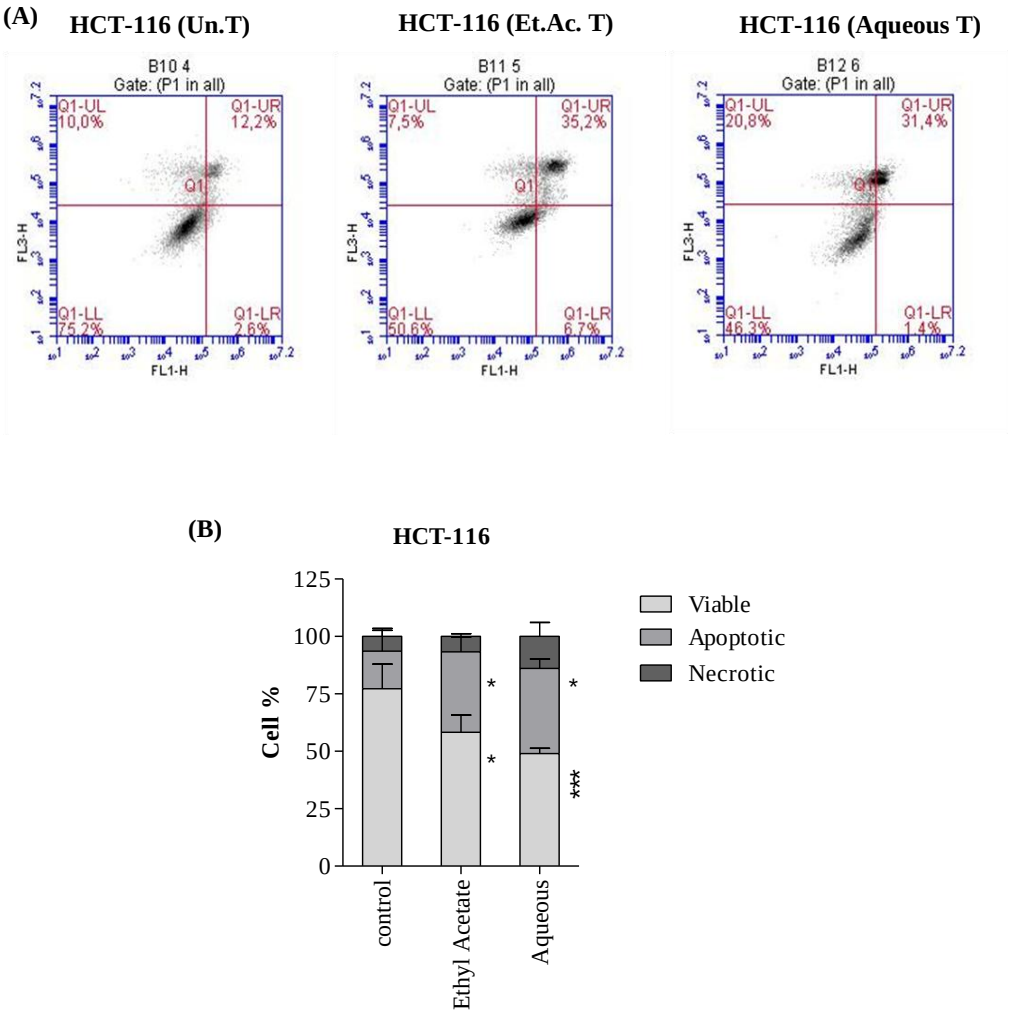


**Figure 3. 7 Effects of ethyl acetate and aqueous fractions on apoptosis in HCT-116 cells by Annexin V staining apoptosis assay.** HCT-116 cells were treated with 200 µg/ml ethyl acetate fraction and 350 µg/ml aqueous fraction for 48 h. 5 mM NaBt was used as a positive control. A) Representative graphs show the pattern of the cell populations in untreated and treated samples. B) Graph shows cell percentage of viable, apoptotic and necrotic populations. Light grey bars indicate viable, grey bars indicate apoptotic and dark grey bars indicate necrotic populations. The assay was repeated three times. Significant differences were compared with the control at *$p < 0.05$ and *** $p < 0.0001$ by one way ANOVA with post hoc Tukey's multiple comparison test.

### 3.4 Effect of the EEB and its Active Fractions on MAPK Pathway

**ERK Pathway**

Our results showed that EEB, as well as its ethyl acetate and aqueous fractions had a significant inhibitory effect on proliferation in different cell lines. To explore the molecular basis of their antiproliferative effects, we investigated one of the best studied signal transduction pathways that plays major roles in cell growth and survival (Fang and Richardson, 2005), the Mitogen Activated Protein Kinase (MAPK) pathway. Activation of the MAPK pathways is a frequent occurrence in several cancers as a consequence of various mutations (Davies et al., 2002; Wang et al., 2004). The HCT-116 cell line has a Ras mutation (Jiang et al., 1989) that results in constitutively active MAPKs. The cell lines were treated with EEB at the predetermined $IC_{50}$ values (300 µg/ml for HCT-116, 250 µg/ml for HT-29, 170 µg/ml for MCF-7 and 400 µg/ml for PC-3) and 48 h treatment duration was used in all assays (Enayat et al., 2013). Treatment of HCT-116 cells with 300 µg/ml of EEB led to a significant decrease in phosphorylation of ERK1/2 (the active form of protein) when compared to the vehicle treated control sample (Figure 3.8 A). As a positive control, the cells were treated with 10 µM of U0126 (specific inhibitor of MEK1/2) for 2 h in serum free medium. An inhibitory effect of EEB on ERK1/2 phosphorylation was also observed in HT-29 (colon cancer) and MCF-7 (breast cancer) cell lines at varying efficiencies. Interestingly, the PC-3 (prostate cancer) cell line did not show any change in ERK1/2 phosphorylation after EEB treatment (Figure 3.8 B). Treatment of HCT-116 and HT-29 cells with 200µg/ml and 350 µg/ml of ethyl acetate fraction respectively also resulted in a decrease in the phosphorylation of ERK1/2. Treatment with the aqueous sub-fraction was applied to only HCT-116 cells and also showed a robust inhibitory effect on activation of ERK 1/2 compared to vehicle treated control cells (Figure 3.8 C). HT-29 cells were not treated with the aqueous fraction since no significant anti proliferative effect was observed with the aqueous fraction in this cell line with the same range of concentrations.

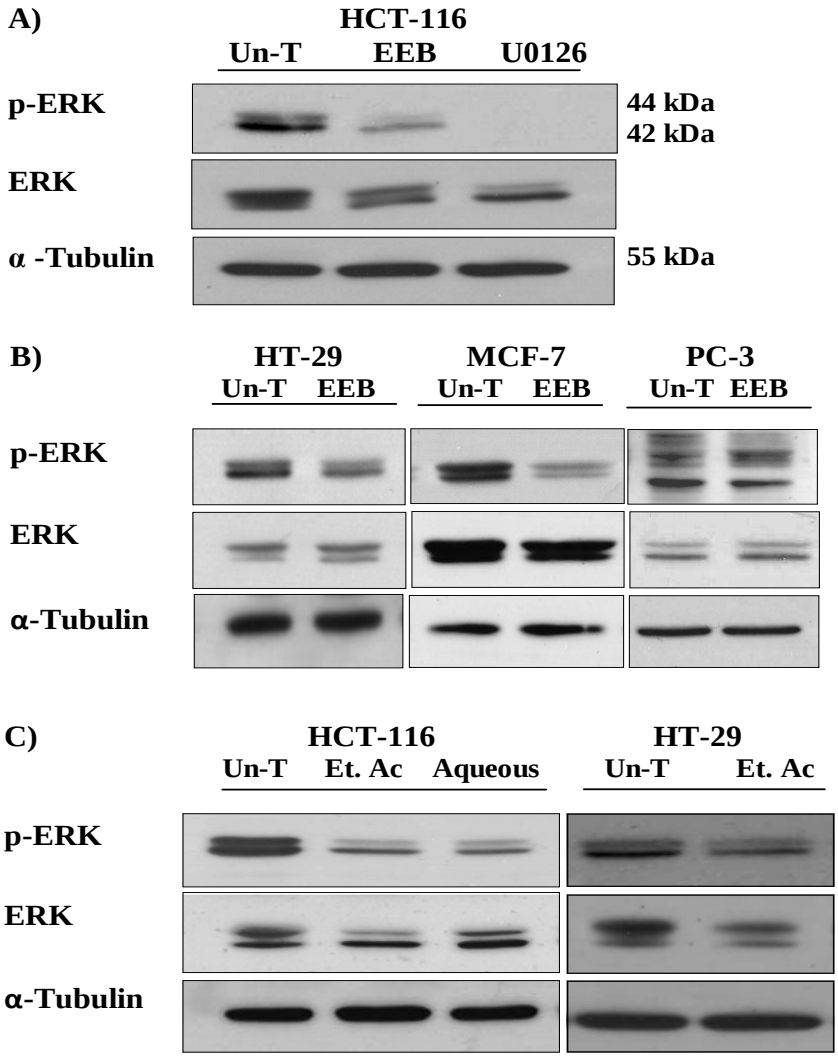


**Figure 3. 8 Effects of EEB, ethyl acetate and aqueous fractions on ERK/MAPK pathway in different cancer cells were determined by western blotting assay.** A) Western result showed inhibitory effect of (300 μg/ml) EEB treatment for 48 h on phosphorylation of ERK in HCT-116 cells. U0126 is a specific inhibitor of MEK1/2. B) Reduction of phosphorylated ERK by EEB treatment in different cancer cell lines. HT-29, MCF-7 and PC-3 cells were treated with 250 μg/ml, 170 μg/ml and 400 μg/ml of EEB for 48 h, respectively. C) Reduction of phosphorylated ERK by treatments of (200 μg/ml) ethyl acetate and (350 μg/ml) aqueous fractions in HCT-116 and treatments of (350 μg/ml) ethyl acetate fraction in HT-29 cells after 48 h treatment. α-Tubulin was used as a loading control. The assay was repeated three times independently.

**JNK and p38 Pathways**

The JNK and p38 proteins are the other important members of MAPK pathways that are also known as stress activated kinases. Abnormal regulation of JNK and p38 have been implicated in some cancers (Wagner and Nebreda, 2009). These pathways are activated mainly by cytokines and ROS (Uehara et al., 2005). Previously, our group showed that ROS levels were decreased after treatment of cells with EEB (Enayat et al., 2013). In order to explore these important pathways, western blotting analysis was carried out. Treatment with EEB led to a reduction in the phosphorylated levels of both p38 and JNK compared to vehicle treated control cells (Figure 3.9 A). The decreased level of phosphorylated JNK and p38 therefore were corroborated with the reductions of ROS level observed after EEB treatment (Enayat et al., 2013). However, treatments of ethyl acetate and aqueous fractions did not cause any inhibition of p38 (Figure 3.9 B) showing that EEB most likely contained some constituents that specifically inhibited the phosphorylation of p38 and JNK, which were lost during the fractionation of the extract.

The phosphorylated p38 was observed as two bands on the Western blot. To discriminate between phosphorylated p38 and the nonspecific bands, protein isolation was done by lysis buffer in the absence of phosphatase inhibitor, which allowed phosphatases to dephosphorylate the phospho-proteins. A loss of the lower band in the Western blot indicated that it was the specific band for phosphorylated p38 (Figure 3.9 C).

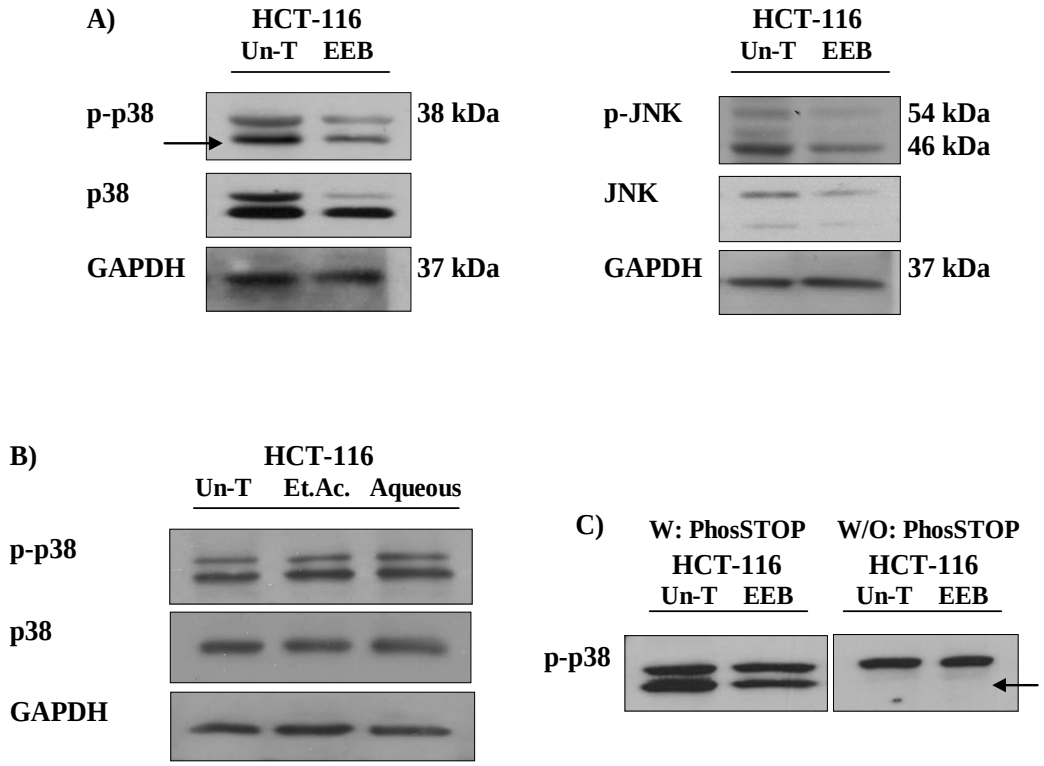


**Figure 3. 9 Effects of EEB, ethyl acetate and aqueous fractions on SAPK/JNK and p38/MAPK pathways were determined by western blotting assay.** A) Inhibitory effect of (300 μg/ml) EEB treatment on phosphorylation of p38 and JNK in HCT-116 cells B) Phosphorylated p38 levels after treatments of (200 μg/ml) ethyl acetate and (350 μg/ml) aqueous fractions in HCT-116 cell line. C) Identification of the phosphorylated p38 band in the absence of phosphatase inhibitor. Arrow shows specific location of p-p38 proteins. GAPDH was used as a loading control.

# CHAPTER 4

# DISCUSSION

According to the American Cancer Society, colon cancer has been counted as the second leading cause of cancer-related deaths in the United States and 102,480 new cases of colon cancer as well as 50,830 deaths have been estimated for 2013 (American Cancer Society, 2013 www.cancer.org). Free radicals, both ROS and RNS, are some of the major initiators or promoters of cancer progression through DNA damage, alteration of antioxidant defense system and activation of survival signal transduction pathways (Kumar et al., 2008; Ohnishi et al., 2013; Sun, 1990). Consumption of high amounts of fruits and vegetables are supposed to restrain 20% or more of all cases of cancer, which indicates the importance of diet based cancer prevention (Glade, 1999). Moreover, research on dietary and natural botanical compounds have increased owing to their safety, low toxicity, and general acceptance of dietary supplements (Amin et al., 2009).

Willow bark has been consumed as a herbal medicine against various diseases for several centuries. The high salicin contents of Willow bark provides analgesic properties, and has been included in the German Pharmacopeia as an agent to reduce fever, rheumatic ailments and headaches (Schmid et al., 2001). Enayat et al. showed that ethanolic extract from bark (EEB) of *Salix aegyptiaca* did not have any significant antiproliferative effect on non-transformed colon fibroblast cell line; on the other hand, robust growth inhibitory effect was observed with colon cancer cell lines (Enayat et al., 2013). Moreover, Willow bark extract is commercially available

in tablet, powder and liquid forms through various online stores. However, there is insufficient information about the effects of Willow bark extract at the molecular level. In this study, the effects of the ethanolic extract from the bark of *Salix aegyptiaca* was investigated on the colon cancer cell line HCT-116. We first analyzed the antioxidant capacity of the extract followed by investigations on the effect of the extract on the MAPK pathway.

### 4.1 Determination of Free Radical Scavenging Activity

The free radical scavenging activities of EEB and its fractions were determined by the DPPH assay that indicates the radical quenching ability of the antioxidant compounds. $IC_{50}$ values were calculated at two time points; 30 min and 1 h. A lower $IC_{50}$ value indicates a higher antioxidant capacity of the compound. Our results showed that the highest free radical scavenging activity was detected in the ethyl acetate fraction at both 30 min and 1 h time points (13 ± 1 μg/ml and 11 ± 1 μg/ml, respectively) compared to EEB (23 ± 1.5 and 21 ± 1 μg/ml, respectively) and the aqueous fraction (38 ± 2 μg/ml and 33 ± 2 μg/ml, respectively). Moreover, we detected considerably higher $IC_{50}$ values (indicating lower radical quenching properties) from the fractions in n-hexane and n-butanol (56 ± 3 μg/ml and 52 ± 2 μg/ml at 30 min and 47 ± 2 μg/ml and 45 ± 1 μg/ml at 60 min, respectively, Figure 3.1). The n-hexane and n-butanol fractions did not show any significant antioxidant activity, thus we omitted these fractions from further experiments. BHT is a synthetic antioxidant compound that is used as a standard in antioxidant experiments. The $IC_{50}$ value of BHT was reported as 26 μg/ml (Enayat and Banerjee, 2009). Therefore, the ethyl acetate fraction and EEB showed higher scavenging activities than a synthetic antioxidant and therefore they could be an alternative to synthetic antioxidants.

## 4.2 Determination of Total Phenolic and Total Flavonoid Content

The total phenolic content of EEB and its active fractions were determined by the Folin-Ciocalteu method. Standard curves were generated with different concentrations of gallic acid (a commercially available phenolic compound) for each independent experiment and the total phenolic content was calculated from the equation generated from the curve. The highest total phenolic content was observed in the ethyl acetate fraction (29.6 ± 2 mg GAE/100 g dried weight) followed by EEB (25 ± 1.5 mg GAE/100 g dried weight) and the aqueous fraction (7.8 ± 1 mg GAE/100 g dried weight) (Figure 3.2). Our results indicated that most of the phenolic compounds were found in ethyl acetate fraction, which may have resulted from the polarity of ethyl acetate as a solvent. Fruits, vegetables, herbs, cereals, seeds that form a part of our diet, include several phenolic compounds acting as natural antioxidants and can neutralize the negative effects of free radicals (Milbury and Richer, 2008).

The total flavonoid content of EEB and its active fractions were determined by the aluminum chloride colorimetric method. Various concentrations of EGCG (a commercially available flavonoid) were used to generate a standard curve. A new standard curve was generated for each independent experiment and total flavonoid contents were calculated from the equations of the graphs. Our results showed that EEB possessed the highest total flavonoid content (47.5 ± 2 mg of EGCG equivalent/100 g dried weight) followed by ethyl acetate (29.5 ± 1.5 mg of EGCG equivalent/100 g dried weight) and aqueous fractions (10.4 ± 1 mg of EGCG equivalent/100 g dried weight) (Figure 3.3). Flavonoids have beneficial effects in several diseases; however, poor absorption rate and quick excretion of flavonoids may lead to low availability in the body, which restricts their antioxidant activity. Therefore, consumption of flavonoid-rich foods can be helpful to increase the availability of flavonoids. Moreover, it has been shown that flavonoids have anticarcinogenic effect owning to its protective ability against ROS damage (Yang et al., 2001).

### 4.3 Antiproliferative Effects of EEB and its Fractions

Antiproliferative effects of EEB and its fractions were shown by the MTT and BrdU cellular proliferation assays. Results showed that treatment with the extract led to reduction of growth inhibition in both HCT-116 and HT-29 cell lines. $IC_{50}$ values of EEB, ethyl acetate and aqueous fractions with HCT-116 cells were 275 ± 9 μg/ml, 192 ± 5 μg/ml and 190 ± 5 μg/ml, respectively (Figure, 3.5). $IC_{50}$ values of EEB, and ethyl acetate fractions on HT-29 were 295 ± 19 μg/ml, 227 ± 25 μg/ml, respectively. There was no significant inhibition on proliferation when the HT-29 cells were incubated with the aqueous fraction (Figure 3.4). Enayat et al. reported the inhibitory effects of EEB and its fractions on MCF-7 breast and PC-3 prostate cancer cells and their $IC_{50}$ values were determined as 170.3 ± 1.7 μg/ml (in MCF-7) and 427 ± 17 μg/ml (in PC-3) for EEB, 157.9 ± 2.5 μg/ml (MCF-7) and 206.4 ± 9 μg/ml (in PC-3) for ethyl acetate fraction, 181.9 ± 1.6 μg/ml (in MCF-7) and 556 ± 61 μg/ml (in PC-3) for the aqueous fraction. Other fractions (n-hexane and n-butanol) were screened by MTT assay and no inhibitory effects were observed after these treatments. EEB, ethyl acetate and aqueous fractions were therefore selected for the subsequent experiments (Enayat et al., 2013).

In order to determine the active compounds that were responsible for the inhibitory effect on proliferation, tandem mass spectroscopy (MS-MS) was carried out with the ethyl acetate and aqueous fractions. Several phenolic compounds (gallic acid, p-coumaric acids) and flavonoids (catechin, quercetin, apigenin) were found in fractions and their equivalents in EEB were calculated. To detect which of these active constituents contributed towards the antiproliferative property of EEB, MTT assays were carried out with commercially available catechin, catechol and salicin compounds. Surprisingly, the results showed no significant decrease in proliferation for each individual treatment. HCT-116 cells were then treated with a combination of catechin and catechol compounds to determine whether a mixture of these phytochemicals led to the inhibition of proliferation on cancer cells. The results revealed that treatment of HCT-116 cells with a combination of catechin and

catechol had a significantly more potent effect on growth inhibition than the individual compounds (Figure 3.6). This may be due to synergistic or additive interactions between catechin and catechol. Therefore the crude extract from willow bark with its combination of various phytochemicals may be more beneficial for general health and curative effect on diseases.

We know that EEB is has several constituent phenolic compounds and flavonoids (Enayat and Banerjee, 2009). These compounds have ability to neutralize the negative effects of free radicals that makes them potent chemopreventive agents to diseases that result from oxidative stress.

### 4.4 Effect of the Active Fractions on Apoptosis

Since loss of proliferation is frequently accompanied by the induction of apoptosis, and EEB was shown to induce apoptosis in colon cancer cell lines (Enayat et al., 2013), we next determined whether apoptosis was induced in cells treated with the fractions of EEB. Annexin V staining assay was carried out at the end of treatment of HCT-116 cells with ethyl acetate (200 μg/ml) and aqueous fractions (350 μg/ml) for 48h. The results indicated that the ethyl acetate and aqueous fractions resulted in significantly increased apoptotic populations when compared to the untreated samples. Higher necrotic cell populations were observed in the cells treated with the aqueous fraction when compared to the cells treated with the ethyl acetate fraction most likely due to the unique combinations of constituent phytochemicals in each fraction.

## 4.5 Effect of the EEB and Active Fractions on MAPK Pathways

It has been shown that some flavonoids can decrease the proliferation of cancer cells via the inhibition of the transcriptional activity of Protein Kinase C (PKC) and Activating Protein -1 (AP-1) (Milbury and Richer, 2008). Consequently, we next examined the hypothesis that a mixture of natural compounds present in EEB and its fractions could have led to a reduction of proliferation through their effects on signal transduction pathways in cancer cells.

### ERK Pathway

Mitogen-activated protein kinases (MAPK) are a group of proteins that are conserved from yeast to humans and regulate several critical proteins that are important in cellular processes such as those involved in proliferation, apoptosis, differentiation and cell survival (Garavello et al., 2013; Madhala-Levy et al., 2012; Roy et al., 2010 and Widmann et al., 1999). Activation of the MAPK signaling pathway has been reported in various cancers (Adeyinka et al., 2002; Brose et al., 2002; McCubrey et al., 2007). Therefore the inhibition of MAPK pathways as a target of cancer therapy has also been extensively studied (Engelman et al., 2008; Sebolt-Leopold, 2004). Members of the MAPK cascade are important targets for anticancer drugs due to their central roles in signal transduction networks (Santarpia et al., 2012). In this study, we explored the effect of EEB on the activation of ERK1/2 in different cell lines. Results showed that after treatment of cancer cells with EEB for 48 h phosphorylation of ERK1/2 (the active form of proteins) was decreased when compared to the untreated samples for HCT-116, HT-29 and MCF-7 cell lines. However, there was no significant reduction of phosphorylated ERK1/2 levels in PC-3 cells. It has previously been reported that the active form of ERKs were found at low levels in PC-3 cells (McCubrey et al., 2007). As a positive control, cells were treated with 10 μM of U0126 (MEK inhibitor) that resulted in inhibition of MEK and a dramatic decreased in phosphorylation of ERK1/2 levels (downstream target of

MEK) (Figure 3.8). Western blotting assays carried out using lysates from HCT-116 cells treated with both ethyl acetate and aqueous fractions indicated a noteworthy decrease in the phosphorylated form of ERK1/2. For HT-29 cells, treatment was carried out only with the ethyl acetate fraction treatment since the aqueous fraction did not show any alteration in the proliferation of these cells. Again, the extent of inhibition of ERK1/2 phosphorylation when HT-29 cells were treated with the ethyl acetate fraction was minimal when compared to HCT-116 cells. These variations in the efficacy of the ethyl acetate fraction on HCT-116 and HT-29 cells may stem from the different mutations found in these cell lines (Shakoori et al., 2005).

**JNK and p38 Pathways**

JNK and p38 pathways are two other major members of MAPK pathways that are also termed as stress activated protein kinase pathways. JNK and p38 pathways are activated by growth factors, cytokines, oxidative stress, ER stress, UV exposure and DNA damage (Hayakawa et al., 2012; Zarubin and Han, 2005) Activation of JNK and p38 pathways have been implicated in various cancers such as liver cancer (Sakurai et al., 2006), lymphoma (Elenitoba-Johnson et al., 2003), breast cancer (Esteva et al., 2004) and prostate cancer (Vivanco et al., 2007). Western blotting analysis showed that treatment with EEB resulted in a reduction of the phosphorylated levels of both p38 and JNK in HCT-116 cells (Figure 3.9 A). As the phosphorylated p38 band appeared as a doublet, identification of the specific phospho-p38 band was carried out by isolating protein lysates from the cells with a lysis buffer that lacked phosphatase inhibitor (Figure 3.9 C). The loss of the lower band in the absence of phosphatase inhibitors indicated that band to be the specific phospho-p38 band. One of the major downstream targets of JNKs is the c-jun protein whose activation leads to formation of active AP-1 complex that induces proliferation in cancer cells (Loo, 2003; Turpaev, 2006). Moreover, it has also been shown that the down regulation of JNK1 can decrease the expression of Myc and increase the expression of the CDK inhibitor p21 in hepatocellular carcinoma (HCC) cells (Hui et al., 2008; Wagner and Nebreda, 2009). Additionally, the p38 pathway

has been shown to regulate the production of pro-inflammatory cytokines (COX-2, TNFα and IL-1). As it is well known that chronic inflammation promotes carcinogenesis, the inhibition of p38 activity in cancers related to chronic inflammation (such as colon cancer and HCC) might be important for therapeutic purposes (Loo, 2003).

Our group has previously shown that treatment with EEB led to a decrease in the MMP proteins in cancer cells (Enayat et al., 2013). Activation of AP-1 and p38 may lead to the stabilization of matrix metalloproteinases, which in turn may be important for the invasive properties of these cells (Reunanen et al., 2002).

We have also shown that treatment with EEB resulted in a reduction of superoxide dismutase activity in cancer cells (Enayat et al., 2013). Therefore the decreased activation of stress activated kinases when cells are treated with EEB corroborates to an inhibition of the generation of ROS in cancer cells. Interestingly, the ethyl acetate and aqueous fractions did not inhibit phosphorylation of p38 (Figure 3.9 B). These data indicate that EEB includes a mixture of compounds that perhaps were lost from the fractions during the process of fractionation.

# CHAPTER 5

# CONCLUSION

Free radicals and antioxidants are involved in oxygen homeostasis that play crucial roles in cellular functions. Oxidative stress is enhanced when free radicals are formed and antioxidants can ameliorate damage that result from oxidative stress. Therefore antioxidants are vital components in oxygen metabolism. Different fruits, vegetables, and other plant based diets provide various phenolic compounds and flavonoids that contribute general health improvement and protection against many diseases due to their antioxidant properties.

*Salix aegyptiaca* is an herbal medicine that is used as a painkiller due to its high constituent salicin. It also contains plenty of phytochemicals, phenolic compounds, and flavonoids (Enayat et al., 2013). The objective of this study was to explore antioxidant and anticarcinogenic properties of the ethanolic extract of bark (EEB) from *Salix aegyptiaca* and its fractions. For this purpose, free radical scavenging activity of EEB and its four fractions (ethyl acetate, aqueous, n-hexane and n-butanol) were detected by DPPH assay and the highest scavenging activity was observed in the ethyl acetate fraction. Total phenolic and flavonoid contents of EEB, ethyl acetate and aqueous fractions were determined and the highest phenolic compounds were found in the ethyl acetate fraction whereas the highest flavonoid content was present in EEB. Treatments of cancer cell lines with EEB, ethyl acetate and aqueous fractions resulted in a dose dependent loss of proliferation. Tandem mass spectrometry of the ethyl acetate and aqueous fractions indicated that catechol,

catechin and salicin were the major constituents (Enayat et al., 2013). Interestingly, proliferation assays carried out with commercially available pure catechol, catechin and salicin showed that a combination of catechol and catechin (as opposed to the individual pure compounds) resulted in synergistic or additive inhibitory effects on proliferation.

Along with EEB (Enayat et al., 2013) its active fractions (ethyl acetate and aqueous) had ability to induce apoptosis in HCT-116 cells explaining the loss of proliferation. To understand the mechanism behind the loss of proliferation and induction of apoptosis, members of MAPK signaling pathway was investigated. We have observed the treatment of cells with EEB as well as its fractions decreased the phosphorylation, and thereby the activation of ERK1/2. However, only treatment with EEB and not its fractions resulted in the inhibition of phosphorylation of p38. These data show that EEB may interfere with the MAPK pathway; one of the most commonly mutated and deregulated mitogenic pathways in cancer cells.

Based on our data, we propose that the mixture of various bioactive compounds found in EEB contributed towards its anticarcinogenic characteristics. Cancer cells can develop resistance to single compound and also multiple mutations in cancer cells that leads to abnormality in various different signaling pathways. Hence, combination of multiple compounds will show better efficacy than a single compound. We propose that one of the ways by which EEB may exert its chemopreventive action is through the inhibition of the MAPK pathway and therefore can be consumed for health benefits.

## 5.1 Future Perspectives

Since EEB is a crude mixture of various different compounds, it is necessary to carry out an HPLC fingerprint of the different constituents. Moreover, specific downstream targets of MAPK pathways can be explored to further understand the mechanism of action of the extract in cancer cells. In vivo experiments using various different models to induce colon cancer and then treatment with EEB will also elucidate the efficacy of the extract.

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## APPENDIX A

## DETERMINATION OF FREE RADICAL SCVENGING ACTIVITY OF ETHANOLIC EXTRACT OF BARK FROM *S. AEGYPTIACA*

$$\% \text{ of DPPH radical scavenging Activity} = \left(\frac{\text{Absorbance of DPPH-Absorbance of Sample}}{\text{Absorbance of DPPH}}\right) x\ 100$$

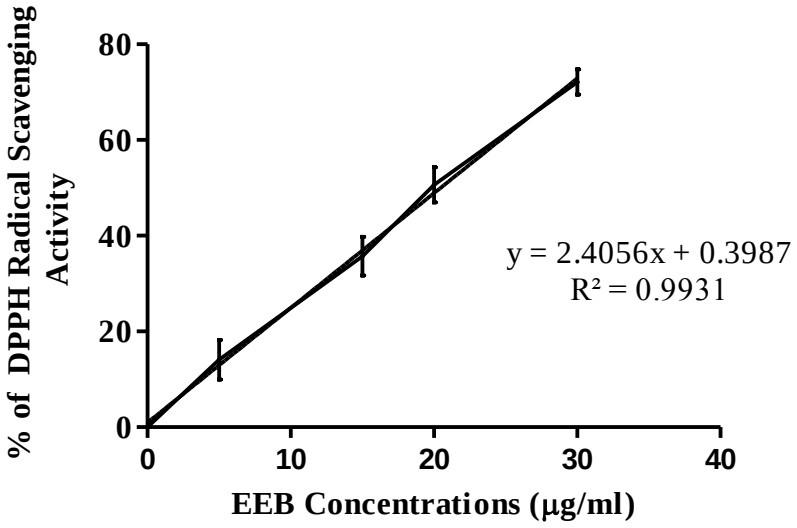


**Figure A. 1** Free radical scavenging activity (%) of EEB with different concentrations at 60 min. Equation was used to calculate $IC_{50}$ value of sample.

Free radical scavenging activity (%) vs concentration graph was generated for each replicates of EEB and its fractions. Equations generated from graphs were applied to

each replicates of all samples and $IC_{50}$% values were determined as the mean of these replicates.

$y = 2.4056x + 0.3987$

y=50 (50% scavenging activity)

$IC_{50}$ % of EEB = (50-0.3987)/2.4056

= 20.62 μg/ml

## APPENDIX B

## GALLIC ACID STANDARD CURVE TO DETERMINE TOTAL PHENOL CONTENT

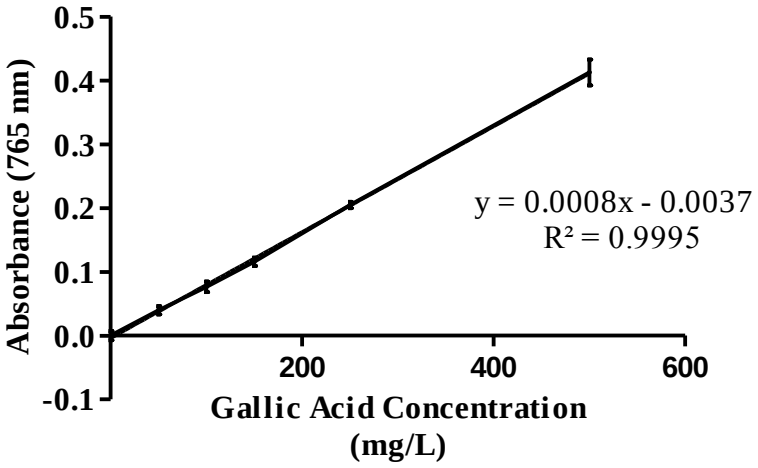


**Figure B. 1** Gallic acid standard curve. Equation was used to determine gallic acid equivalents of sample. New standard curve was generated for each assays

## APPENDIX C

## EGCG STANDARD CURVE TO DETERMINETOTAL FLAVONOID CONTENT

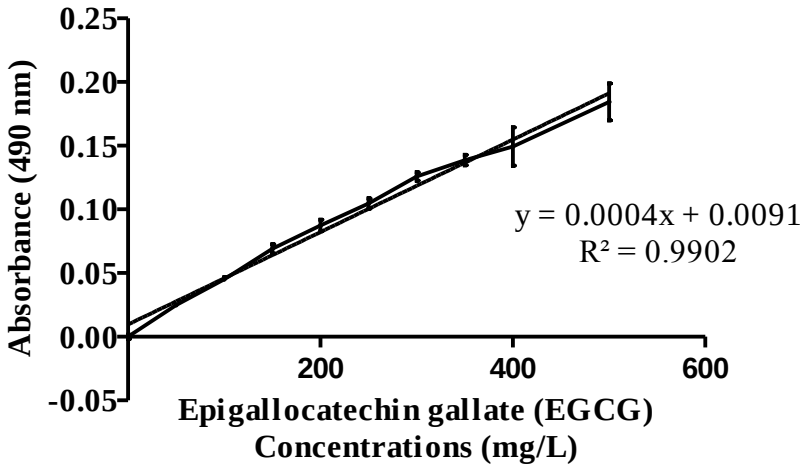


**Figure C. 1** EGCG standard curve. Equation was used to determine EGCG equivalents of samples. New standard curve was generated for each assays.

## APPENDIX D

### BSA STANDARD CURVE TO DETERMINE PROTEIN CONCENTRATION

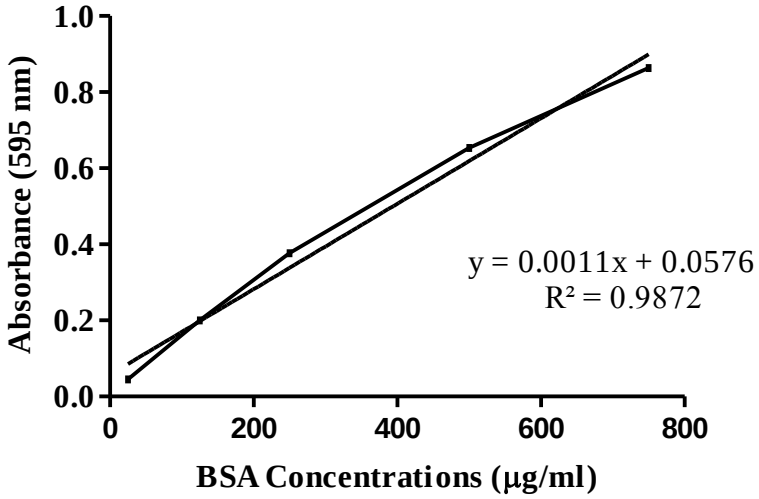


**Figure D. 1** BSA standard curve. Equation was used to determine protein concentrations of samples.

## APPENDIX E

## PRESTAINED PROTEIN LADDER

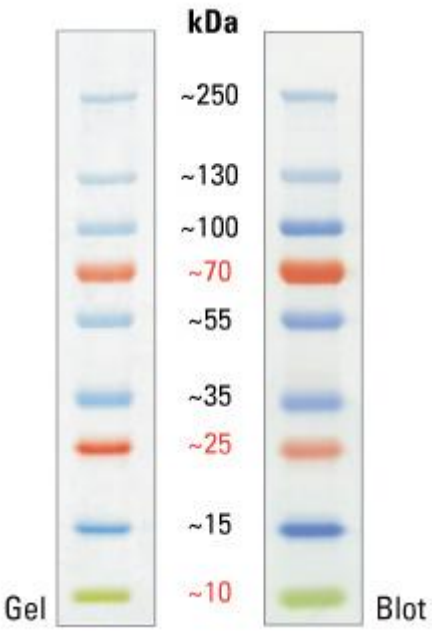


**Figure E. 1** Band profile of the Thermo Scientific PageRuler Plus Prestained Protein Ladder